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Investigation of the Arabidopsis nonhost resistance mechanism against the soybean pathogen, *Phytophthora sojae*

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Investigation of the Arabidopsis nonhost resistance mechanism against the soybean pathogen, *Phytophthora sojae*

by

Rishi Sumit

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Major: Molecular, Cellular and Developmental Biology

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Ames, Iowa

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ABSTRACT

Nonhost resistance (NHR) provides immunity to all members of a plant species against all isolates of a microorganism that is pathogenic to other plant species. Three *Arabidopsis thaliana* *PEN* (penetration deficient) genes, *PEN1*, 2 and 3 have been shown to provide prehaustorial NHR against the barley pathogen *Blumeria graminis* f. sp. *hordei*. *Arabidopsis pen1-1* mutant is penetrated by the hemibiotrophic oomycete pathogen, *Phytophthora sojae* that causes root and stem rot disease in soybean. The *P. sojae* susceptible (*pss*) 1 mutant is infected by both *P. sojae* and the hemibiotrophic fungal pathogen, *Fusarium virguliforme* that causes sudden death syndrome in soybean. Thus, a common *Arabidopsis* NHR mechanism is functional against both hemibiotrophic oomycete and fungal pathogens of soybean. *PSSI* encodes a glycine-rich protein (GRP), named GRP1, with no known function. Transformation of the soybean cultivar Williams 82 with *AtGRP1* conferred enhanced resistance to both *P. sojae* and *F. virguliforme*. My study established that nonhost resistance genes are ideal for engineering broad-spectrum disease resistance in crop plants.

CHAPTER 1. GENERAL INTRODUCTION

Overview of plant nonhost disease resistance

Soybean is one of the most economically important crops in the U.S. and one of the major legume crops worldwide. Soybean is a rotation crop with corn, fixing nitrogen through its association with *Rhizobium* bacterium. It is an important source of proteins and oil for both human consumption and animal feed. Soybean is also used for biofuel production. However, it fails to express full yield potentiality due to attack by various pathogens. For example, the total soybean yield suppression due to pathogens in the United States during the year 2010 was valued at \$5.59 billion. Root and stem rot disease caused by the oomycete; *P. sojae* is one of the most devastating diseases in soybean. The annual losses due to this disease have been estimated to be about 300 million dollars [1].

Nonhost resistance

Plants are exposed to innumerable pathogenic organisms. However, because of various immunity mechanisms, only a few are able to infect and cause diseases in a crop species. There are two main types of plant resistance mechanisms. The cultivar- or race-specific resistance is defined as the resistance when specific members of the species are resistant to a pathogen. Resistance governed by a few members of a crop species is race-specific, which means resistance is good only against a few members of a pathogen. Expression of cultivar- or race-specific resistance is characterized by hypersensitive-response (HR), which is a programmed cell death (PCD) response of plant cells. It is induced by recognition of the invading pathogen races or isolates by host cells. This recognition process is explained by the gene-for-gene hypothesis put forward by Flor.

According to this hypothesis for each gene conditioning resistance in the host there is a corresponding pathogenicity gene in the pathogen [2]. Host resistance against the pathogenic *Avirulence (Avr)* genes is encoded by *Resistance (R)* genes is effective only against a small number of pathogen strains or isolates. On the other hand, nonhost resistance (NHR) is the form of resistance exhibited by all members of a plant species against non-adaptive pathogens [3-4]. Recognition of pathogen associated molecular patterns (PAMPs) of non-adaptive pathogens by nonhost receptors triggers the PAMP-triggered immunity (PTI) in nonhost species [5]. NHR is a less understood plant immunity mechanism. The main causes of NHR were thought to be incompatibility of non-adapted pathogen with the physiology of nonhost plants and inability of non-adaptive pathogens to overcome the plant defenses [6]. The first gene known to confer Arabidopsis NHR against a non-adaptive bacterial pathogen, *Pseudomonas syringae* pv. *phaseolicola* is *NONHOST1 (NHO1)* that encodes a glycerol kinase [7-8]. *NHO1* has been shown to confer nonhost resistance against the bacterial pathogen, *Pseudomonas syringae* pv. *tabaci* and the fungal pathogen, *Botrytis cineria* [7-8]. *NHO1* is an essential component also of the nonhost resistance against *Peronospora trifolium* and *Xanthomonas oryzae* pv. *oryzae*. [7].

NHR acts in two layers against the biotrophic fungal pathogens [9-10]. The first layer of NHR suppresses the invasion by non-adaptive pathogens at the pre-haustorial level. Three NHR genes, *PEN1*, *PEN2* and *PEN3* (for *PENETRATION*), required for penetration resistance of Arabidopsis against the non-adaptive barley biotrophic fungal pathogen, *Blumeria graminis* f. sp. *hordei* have been isolated [9-11]. These genes act at the prehaustorial stage of the pathogen invasions [12]. *PEN1* encodes a soluble N-

ethylmaleimide sensitive attached receptor (SNARE) protein, which is involved in vesicle fusion and secretion of toxic free radicals to the pathogen infection sites [11]. *PEN1* has been localized to the plasma membrane and is responsible for timely assembly of papillae at the site of fungal infection and membrane fusion during fungal attack. The *pen1-1* mutants have been found to be delayed in this function and delayed papillae formation [11]. *PEN1* is a functional homologue of the barley *ROR2*, which is required for basal defense against the powdery mildew pathogen *Blumeria graminis* f. sp. *hordei*. Both *ROR2* and *PEN1* encode a syntaxin protein SYP121. This data thus suggests the mechanistic overlap of nonhost and basal resistance in plants [11]. *PEN2* encodes a glycosyl hydrolase, which has been localized to the peroxisomes [9]. *PEN3* encodes an ATP-binding cassette (ABC) protein of the plasma membrane [10]. *PEN-3* is localized to plasma membrane and helps in transporting the antifungal, toxic compounds to the site of fungal invasion [10]. Cytological studies have demonstrated that *PEN2* and *PEN3* work together to generate and transport toxic chemicals into the infection sites [13]. The first layer of NHR prevents the biotrophic fungal pathogens from penetration and development of feeding structures, haustoria.

Fungal pathogens that overcome the first layer of NHR encounter a post-haustorial defense mechanism, which can be considered as the second layer of NHR. Some of the genes involved in this second layer of Arabidopsis NHR are *EDS1* (*ENHANCED DISEASE SUSCEPTIBILITY 1*), *PAD4* (*PHYTO ALEXIN DEFICIENT4*) and *SAG101* (*SENESCENCE ASSOCIATED GENE 101*) [9]. The *EDS1*, *PAD4* and *SAG101* are all also involved in the expression of cultivar- or race-specific resistance through the salicylic acid defense pathway against biotrophic pathogens. They were also

recently found to form a ternary complex in response to infection by a viral pathogen. It has been showed that *EDS1* interacts with *PAD4* and *SAG101* both separately and as a ternary complex of *SAG101-EDS1-PAD4*. The *EDS1-PAD4* complex is localized to the cytosol while the *EDS1-SAG101* and the *SAG101-EDS1-PAD4* complexes are both localized to the nucleus. [14]. The *sag101pad4* double mutant, but not the *sag101* or *pad4* single mutants are compromised in NHR to *Pseudomonas syringae* pv. *tomato*, thus depicting their functional redundancy [15]. Furthermore, it has been shown that the post-haustorial stage of NHR mechanism is most important in sow thistle for providing resistance against a poorly adapted powdery mildew fungus *Golovinomyces cichoracearum* *UMSG1* [16]. A similar mechanism could also be important for the NHR of Arabidopsis against the non-adapted oomycete pathogen, *P. sojae*. *R* genes regulate various disease resistance pathways through signaling components. Four well characterized resistance pathways are regulated by salicylic acid (SA), jasmonic acid (JA), ethylene and nitric oxide [17]. Disruptions in the SA pathway in Arabidopsis *sid2* mutant or in transgenic Arabidopsis by introducing bacterial *NahG* encoding salicylate hydroxylase allows *Uromyces vignae*, a nonhost pathogen to establish feeding structures called haustoria. Arabidopsis *jar1* mutants, lacking a functional pathway regulated by JA allow necrotrophic nonhost pathogens to grow in Arabidopsis. This suggests the importance of both SA and JA in providing Arabidopsis nonhost resistance against *U. vignae* [18]. Specific genes such as the *PR-1* (*Pathogenesis Related-1*), and *PDF1.2* (*Plant Defensin Factor 1.2*) are known as markers for the regulation of the salicylic acid and jasmonate pathways of plant defense respectively. These genes have been used in several studies to understand the regulation and inter-relation of these opposing disease

resistance pathways in plants. Also, the *Ethylene Response Factor -1 (ERF-1)* is a marker gene used to gauge the regulation of the ethylene resistance pathway. The bulked segregant analysis has been previously applied as a rapid method to map and eventually identify a gene through the forward genetic screening method in which the phenotype observed is eventually assigned to a yet unknown gene causing that phenotype [19]. The bulked segregant analysis includes a F₂ population which is segregating for the phenotype in a single gene effect of Mendelian ratio. Single sequence length polymorphism (SSLP) markers showing polymorphism between the most commonly used ecotypes of *Arabidopsis*, the Columbia-0 and *Landsberg erecta (Ler)* are available in the scientific community. Also, this knowledge has been extended to other ecotypes of the model plant, *Arabidopsis thaliana*. For the specific objective of this proposed study, the method includes identifying *Arabidopsis* mutants, which show enhanced susceptibility to the soybean oomycete pathogen, *P. sojae* as a symptom showing loss or breakdown of the *Arabidopsis* nonhost resistance against this pathogen. There have been several other studies that successfully used the bulked segregant analysis method to identify resistance genes from various plant systems through a forward genetics approach [20-21]. This establishes the feasibility of using this rapid method towards isolation of a single resistance gene from a plant species through a forward genetics approach.

***Arabidopsis – Phytophthora sojae* interaction**

Phytophthora sojae is one of the most destructive pathogens of soybean [1]. The pathogen, formerly known as *Phytophthora megasperma* f. sp. *glycinea*, causes both pre-emergence and post-emergence damping off in the seedlings [22]. The pre-emergence damping off symptoms include rotting of germinating seedlings. If infected seedlings

emerge from soil, the lower taproot becomes soft and brown and discoloration extends to the hypocotyls [22]. The oomycete pathogen typically grows as aseptate hyphae and is heterophylic. It produces different types of asexual spores, namely the zoospores, sporangia and the chlamydospores. Both sporangia and zoospores can produce hyphae under favorable conditions. Although sporangia can germinate to produce hyphae, usually they carry the zoospores. Zoospores have been known to form cysts under unfavorable conditions and these cysts can eventually germinate to produce hyphae [23]. In addition to asexual spores, the pathogen produces sexual spores, oospores, from mating of female oogonia and male antheridia. A series of single race-specific *Rps* genes have been employed for providing immunity to this destructive pathogen in soybean [24].

Arabidopsis is a nonhost for *P. sojae* [25]. *Phytophthora sojae* zoospores usually form cysts upon inoculation on *Arabidopsis* cotyledons. There is also some germination and formation of bulb-like swollen structures (appressorium), which usually grow over the anticlinal walls between epidermal cells. There is seldom any growth through the stomata and rarely haustoria or the feeding structures are formed in the wild type ecotype, Columbia-0 (Col-0) [25]. The haustoria rarely reach mesophyll cells and are never able to colonize the mesophyll cells [26]. There is an active rearrangement of actin microfilaments around the site of fungal penetration in the *Arabidopsis* cotyledons. This is accompanied by deposition of callose or other dense material at the fungal penetration sites [26]. The *PEN1* gene is known for timely assembly of papillae and callose depositions at the fungal penetration sites [27], and thus it is quite likely that I would observe higher proportions of successful penetrations by the fungus in the *pen1-1* mutant lacking PEN1 as compared to the wild type Col-0 plants. *Arabidopsis thaliana* is known

to carry nonhost resistance against the oomycete pathogen *P. sojae* [25]. This nonhost resistance might be due to certain gene or gene products, which are functional in *Arabidopsis* and either absent or non-functional in soybean. There must be certain differences between the nonhost *A. thaliana* and the host plant soybean with respect to the genes and their expression profiles in case of a *P. sojae* attack. These differences make *Arabidopsis* a nonhost and soybean a host to *P. sojae* and these directly relate to the major objectives of this study.

Thesis Organization

This thesis is presented in an alternate format and is composed of five chapters. A general introduction to the plant nonhost resistance and the *Arabidopsis*-*Phytophthora sojae* interaction is given in Chapter 1. Presented in Chapter 2 is a novel technology, published in BMC Genomics journal, for identification, creation and analysis of sequence based polymorphism (SBP) markers to characterize naturally occurring polymorphism between two *Arabidopsis* ecotypes, Columbia-0 and Niederzenz (Nd). I conducted all experiments to collect and characterize all *Arabidopsis* ecotypes to identify Nd as immune to soybean oomycete pathogen, *P. sojae* and conducted the sequence analysis and marker identification with Dr. Binod B. Sahu. The development of this novel marker methodology was important for fine-mapping of the *Phytophthora sojae* susceptible (*PSSI*) gene and is likely to contribute to identification of other *pss* and other genes in *Arabidopsis* and other organisms with a publically available reference genome. In Chapter 3, identification, mapping and characterization of a novel *Arabidopsis* nonhost resistance gene, *Phytophthora sojae* susceptible (*PSSI*) is presented. In this chapter, data relating to 1) screening of 3,500 EMS generated M₂ families to identify thirty putative *pss*

mutants, *pss1* through *pss30*; 2) identification of a novel nonhost gene, *PSSI* which provides a previously unknown form of penetration resistance and is required for Arabidopsis nonhost resistance against *P. sojae* and *F. virguliforme*; 3) mapping of *PSSI* gene and 4) characterization of *pss1* mutant is presented. I conducted all the experiments except EMS generation of M₁ mutants and the induction of *P. sojae* effector genes in *pss1* mutant presented in this chapter. This Chapter is adapted from a manuscript published in BMC Plant Biology journal. The data presented in Chapter 4 relates to the cloning, complementation and characterization of the Arabidopsis nonhost gene *PSSI*, which encodes a glycine rich protein (GRP1). In this chapter, data relating to 1) map- based cloning of *PSSI*, encoding a glycine rich protein (GRP1); 2) complementation of *pss1* with Arabidopsis GRP1 and 3) characterization of GRP1 gene is presented. I, along with Dr. Binod B. Sahu conducted all experiments except soybean transformation presented in this chapter. Chapter 5 is a general conclusion chapter and summarizes all the results from Chapter 2 to Chapter 4.

CHAPTER 2. SEQUENCE BASED POLYMORPHIC (SBP) MARKER TECHNOLOGY FOR TARGETED GENOMIC REGIONS: ITS APPLICATION IN GENERATING A MOLECULAR MAP OF THE ARABIDOPSIS THALIANA GENOME

A paper published in BMC Genomics

Binod B Sahu, Rishi Sumit, Subodh K Srivastava, and Madan K Bhattacharyya

MKB conceived and designed the experiments. SKS conducted bioinformatics analyses and identified sequence reads for selected regions of the genome. BBS and RS analyzed the sequence data, performed experiments and analyzed experimental data. BBS and MKB wrote the manuscript. All authors read and approved the final manuscript.

Abstract

Background: Molecular markers facilitate both genotype identification, essential for modern animal and plant breeding, and the isolation of genes based on their map positions. Advancements in sequencing technology have made possible the identification of single nucleotide polymorphisms (SNPs) for any genomic regions. Here a sequence based polymorphic (SBP) marker technology for generating molecular markers for targeted genomic regions in Arabidopsis is described.

Results: A ~3X genome coverage sequence of the *Arabidopsis thaliana* ecotype, Niederzenz (Nd-0) was obtained by applying Illumina's sequencing by synthesis (Solexa) technology. Comparison of the Nd-0 genome sequence with the assembled Columbia-0 (Col-0) genome sequence identified putative single nucleotide polymorphisms (SNPs) throughout the entire genome. Multiple 75 base pair Nd-0 sequence reads containing SNPs and originating from individual genomic DNA molecules were the basis for

developing co-dominant SBP markers. SNPs containing Col-0 sequences, supported by transcript sequences or sequences from multiple BAC clones were compared to the respective Nd-0 sequences to identify possible restriction endonuclease enzyme site variations. Small amplicons, PCR amplified from both ecotypes, were digested with suitable restriction enzymes and resolved on a gel to reveal the sequence based polymorphisms. By applying this technology, 21 SBP markers for the marker poor regions of the Arabidopsis map representing polymorphisms between Col-0 and Nd-0 ecotypes were generated.

Conclusions: The SBP marker technology described here allowed the development of molecular markers for targeted genomic regions of Arabidopsis. It should facilitate isolation of co-dominant molecular markers for targeted genomic regions of any animal or plant species, whose genomic sequences have been assembled. This technology will particularly facilitate the development of high density molecular marker maps, essential for cloning genes based on their genetic map positions and identifying tightly linked molecular markers for selecting desirable genotypes in animal and plant breeding experiments.

Introduction

Discovery of molecular markers has facilitated mapping of both qualitative and quantitative traits. Tightly linked molecular markers facilitate (i) isolation of the genes encoding these traits and (ii) selection of genotypes carrying the desirable alleles. Several molecular marker technologies such as, RFLP, RAPD, DAF, SSR, SSLP, AFLP, CAPS, SNP have been discovered for molecular mapping experiments [28-33]. Fingerprinting of genotypes for restriction fragment length polymorphisms (RFLPs) has been regarded as

the most sensitive method of genotyping. This procedure, however, requires a large quantity of genomic DNA and use of radioactive probes. In the random amplified polymorphic DNA (RAPD) marker technology, multiple random loci of the genomes are PCR amplified with a single, 10 nucleotide long primer of arbitrary sequence [30]. In DNA amplification fingerprinting (DAF), many loci are PCR amplified with the aid of a single, short arbitrary primer, as short as 5-nucleotides long [31]. Simple sequence repeat (SSR) markers, also known as microsatellite markers, utilize the variation for tandem repeats such as (CA)_n repeats observed between genotypes [34]. Simple sequence length polymorphism (SSLP) markers, similar to SSR markers, are designed based on a unique segment of genomic DNA sequence that contains a simple tandem repeat that distinguishes the genotypes. In Arabidopsis, SSLPs are largely based on the (GA)_n repeats [35]. Cleaved amplified polymorphic sequences (CAPS) markers are designed based on restriction fragment length polymorphisms of PCR amplified fragments, when sequence information of one of the haplotypes is unknown [33].

The high-throughput amplified fragment length polymorphism (AFLP) marker technology combines principles of RFLP and random PCR amplification for rapid identification of molecular loci of the entire genome [28]. AFLP technology is particularly suitable for developing high density molecular marker maps, essential for both map-based cloning of genes and the isolation of molecular markers for selecting desirable genotypes in breeding programs. AFLP technology identifies molecular markers based on a fraction of the restriction fragment length polymorphisms between two genotypes. Restriction site associated DNA (RAD) marker technology, on the other hand, generates markers for all polymorphic sites of a restriction endonuclease between

two genotypes; and thus, it is a very sensitive marker technology for developing a high density molecular map [36].

Polymorphisms detected by various marker technologies stated above have been used to generate molecular marker maps of those species that do not have any genome sequences and physical maps. Since assembled genome sequence of many species are available, and the cost of sequencing has declined significantly with advent of the next generation sequencing technologies, single nucleotide polymorphism (SNP) is becoming the most popular molecular marker [37-39]. However, SNP assays are not always simple or flexible. Here, a strategy of using SNPs for rapid generation of molecular markers, termed sequence based polymorphic (SBP) marker technology is described.

The assembled *Arabidopsis thaliana* genome sequence is selected for this study [40]. Many of the ecotypes of this species are available and have been used in mapping experiments to conduct genetic and biological studies. SNPs among some of the accessions or ecotypes of this model plant species are available [41-42]; at <http://www.arabidopsis.org>. Niederzenz-0 (Nd-0), used for mapping the *Phytophthora sojae* susceptible (*pss*) mutants that are infected by the soybean pathogen, *P. sojae* (R. Sumit, B.B. Sahu and M.K. Bhattacharyya, unpublished), was selected for this study. The *pss* mutants were created in the *pen1-1* mutant of the ecotype, Columbia-0 (Col-0). To facilitate mapping of the putative *PSS* gene loci conferring nonhost resistance of *Arabidopsis* against *P. sojae*, SBP markers were developed as follows. Seventy-five nucleotide long sequencing reads obtained by conducting Solexa sequencing of the Nd-0 genome were compared to Col-0 sequences to identify the SNPs, which were subsequently converted to SBP markers if either of the ecotypes was cut by at least one

restriction endonuclease at the SNP sites. By applying this technology, 21 co-dominant SBP markers were generated for the marker-poor regions of the *Arabidopsis* genome. This novel SBP marker technology should be applicable to any higher eukaryotic species with assembled genome sequences for rapid development of high density molecular marker maps for map-based cloning of genes or identification of suitable molecular markers for selection of desirable genotypes in breeding programs.

Material and Methods

Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* ecotypes, Col-0 and Nd-0, were sown on LC1 soil-less mixture (Sun Gro Horticulture, Bellevue, WA) under 16 h light/8 h dark regime at 21°C with approximately 60% relative humidity. The light intensity was maintained at 120-150 $\mu\text{E}/\text{m}^2/\text{s}$ [43]. Ten days after sowing, the seedlings were transplanted in LC1 mixture. The newly transplanted seedlings were covered with humidity domes for two days and thereafter watered every fourth day. A fertilizer mixture of 15:15:15::N:P:K (1% concentration v/v) was applied to the seedlings seven days after transplantation.

DNA preparation and the whole genome sequencing

Genomic DNA was extracted from *Arabidopsis* by the CTAB method [44]. Either young inflorescence or a rosette leaf was selected for DNA extraction. The Nd-0 genome was sequenced in a Solexa, Illumina sequencing platform at the DNA facility, Iowa State University. The 75 bp Solexa Nd-0 reads were saved as the gsNd database (Accession No. SRA048909.1) for further studies.

Analysis of the raw reads from Solexa Sequencing

The raw 75 bp Solexa reads of the gsNd database were analyzed by the mapping algorithms, Efficient Large scale Alignment of Nucleotide Databases (ELAND), which is built in with the Solexa sequence analysis pipeline of the Illumina sequencer [45]. This program can match a large number of reads against a reference genome sequence; e.g., in this study the Arabidopsis Col-0 genome sequence was used as the reference genome. In order to identify the SNPs from the entire Arabidopsis genome (NCBI_SS#478443777 through 428555842), the 75 bp Solexa sequence reads of Nd-0 were compared to the assembled Col-0 genome sequence (version TAIR10) (ftp://ftp.arabidopsis.org/home/tair/Sequences/whole_chromosomes/) by running the SHORE program [46]. The gsNd database also was used for conducting the BLASTN (bl2seq) search for polymorphic sequences of the marker poor genomic regions.

SSLP and CAPS markers polymorphic between Col-0 and Nd-0

Candidate SSLP and CAPS markers available from the TAIR database were selected to cover the entire genome. Sequence information of primers for SSLP markers were obtained from Bell and Ecker [9] and the Arabidopsis Information Resource (TAIR) database (<http://www.arabidopsis.org>). The chromosome map tool function available at the TAIR database (<http://www.arabidopsis.org/jsp/ChromosomeMap/tool.jsp>) was used to map the physical locations of the markers that showed polymorphisms between the two accessions.

PCR conditions and digestion with restriction endonucleases

The final DNA concentration in PCR was 20 ng/μl. The PCR mixtures contained 2 mM MgCl₂ (Bioline, Taunton, MA), 0.25 μM each of forward and reverse primer, 2

μ M dNTPs and 0.5 U Choice Taq polymerase (Denville Scientific, Inc., Metuchen, NJ). For SBP or SSLP, PCR was conducted at 94° C for 2 min, and then 40 cycles of 94°C for 30 s, 50°C or 55°C for 30 s and 72°C for 30 s. Finally, the mixture was incubated at 72° C for 10 min. For CAPS markers, PCR was conducted at 94° C for 2 min, and then five cycles of 94°C for 30 s followed by decreasing annealing temperatures from 55° C to 50° C (-1°C/cycle) and 72° C for 1 min. Then 35 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min were conducted. Finally, the reaction mixtures were incubated at 72°C for 10 minutes. PCR was carried out in PTC-100 Programmable Thermal Controllers (MJ Research Inc., Waltham, MA). The amplified products were resolved on a 4% (w/v) agarose gel at 8 V/cm. Amplified CAPS and SBP products were digested with the respective restriction enzymes following manufacturer's protocols. The ethidium bromide stained PCR products were visualized by illuminating with UV light.

Results

Generation of a global molecular map for the polymorphic loci of the *Arabidopsis thaliana* ecotypes, Col-0 and Nd-0

Arabidopsis is a nonhost for the soybean pathogen, *Phytophthora sojae*. Several putative *P. sojae* susceptible (*pss*) *Arabidopsis* mutants that are infected by this oomycete pathogen were identified (Sumit et al, 2012). In order to map the putative *PSS* genes that confer nonhost resistance of *Arabidopsis* against the soybean pathogen, *P. sojae*, a global map of the SSLP and CAPS markers that are polymorphic between ecotypes, Col-0 and Nd-0 was generated. A group of 126 simple sequence length polymorphism (SSLP) markers (<http://www.arabidopsis.org>) that mapped evenly throughout the entire genome was investigated for polymorphisms. Of these, 50 SSLPs were polymorphic between the

two ecotypes. A group of 48 cleaved amplified polymorphic sequences (CAPS) markers also were investigated for polymorphisms between the two ecotypes (Table 1). Of these, 18 were polymorphic between the two ecotypes. The map positions of all 67 polymorphic SSLP and CAPS markers are presented in Additional file 1. Phenotypes of these markers are presented in Additional file 2.

Generation of SBP markers for saturating a global genome map in *Arabidopsis thaliana*

The global genome map of SSLP and CAPS was marker poor in some genomic regions (Additional file 1). In order to fill out some of the marker poor regions, single nucleotide polymorphism (SNP)-based molecular markers were generated as follows. First, the Nd-0 genome was sequenced in an Illumina/Solexa genome Analyzer II (GAII) at the DNA facility, Iowa State University. Three genome equivalents of Nd-0 sequence in 75 bp reads then were analyzed to discover SNPs (Accession No. SRA048909.1) between Col-0 and Nd-0 by conducting reference guided sequence analysis for all five chromosomes with the aid of the SHORE program [47].

One can also identify candidate SNPs (NCBI_SS#478443777 through 428555842) for targeted genomic regions by comparing Nd-0 query sequence with Col-0 sequence in batches of ~20 kb (Figure 1). This was achieved by aligning the two sequences using BLAST (bl2seq) program at the NCBI website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). In Solexa sequencing, many sequencing reads could be originated from PCR products of a single DNA molecule (Additional file 3). SNPs originating from 75 bp reads of single PCR molecules are less reliable, because some of such single nucleotide polymorphisms may be generated from PCR-based

mutations. This limitation was overcome by selecting those SNPs that originated from at least two staggered 75 bp sequence reads (Figure 2). Staggered reads are considered to originate from independent DNA molecules. Thus, SNPs observed in at least two overlapping reads with staggered ends are considered most likely authentic and selected for the next step. In parallel, to eliminate any possible SNPs originating from sequencing errors in the publicly available Col-0 sequence, the SNPs containing Col-0 sequences were investigated for possible 100% nucleotide matches with (i) expressed sequence tags (ESTs) or (ii) genomic sequences of at least two BACs (Additional file 4) in GenBank. The Col-0 sequences that met one of these criteria were considered further for SBP marker development. Use of the above two criteria in selecting SNP-containing sequences increased the chance of SBP marker identification. High quality SNPs identified through SHORE analysis could be directly applied for developing SBP markers, if genomes are sequenced to higher depth ($\geq 20X$ genome equivalents).

In the last step of the SBP marker development, SNPs were converted to possible restriction endonuclease site-specific polymorphisms between the Col-0 and Nd-0 haplotypes by analyzing restriction enzyme digestion patterns of the selected Nd-0 and Col-0 sequences using a suitable program (<http://tools.neb.com/NEBcutter2/>). PCR amplicons of approximately 200 nucleotides and that contained variations for restriction endonuclease sites between Col-0 and Nd-0 ecotypes were considered as putative SBP markers. Finally, primers for PCR amplification were designed in such a way that one can easily distinguish the haplotype-specific restriction enzyme length polymorphisms following separation of the restriction enzyme digested PCR products on a 4% (w/v)

agarose gel. Following this protocol, 21 SBP markers for some of the marker poor regions of the *Arabidopsis* genome were identified (Figures 3 and 4; Table 2).

Discussion

The use of molecular markers has gained importance in genetic studies particularly for map based cloning of genes [48]. The relatively low cost of sequencing a genome, with the emergence of high throughput sequencing technology, has facilitated genome wide polymorphism studies [49-50]. The SBP marker technology can convert most of the single nucleotide polymorphisms to molecular markers for any genomic regions. SBP markers developed based on sequence information are ideal for those species, whose genomes are sequenced and assembled. Reference genome sequence can be utilized to develop SBP markers for a specific genomic region with known physical location. Thus, marker-poor regions can be enriched with SBP markers. In this study, the applicability of the SBP marker technology for generating markers is shown for improving a genetic map that represents polymorphisms between two *Arabidopsis* ecotypes, Col-0 and Nd-0 (Figure 4). SBP markers were generated from just three genome equivalents Nd-0 genome sequence of 75 bp Solexa reads. The method also has been successfully applied in developing a high density molecular map of the *PSSI* gene that confers nonhost resistance against the soybean pathogens, *Phytophthora sojae* and *Fusarium virguliforme* (R. Sumit, B.B. Sahu and M.K. Bhattacharyya, BMC Plant Biology).

The SHORE program used in this study is highly powerful and has been employed successfully in identification of a mutation through analysis of deep sequence

data of a bulk of 500 mutant F₂ progenies [51]. If the genome sequencing is not conducted to a higher depth (e.g. ≥ 20 fold), SNPs identified through SHORE analyses can be verified by conducting BLAST analyses. Staggered Solexa sequence reads (Figure 2) containing SNPs are considered for generating SBP markers for such a scenario. Similarly, candidate SNP containing regions of the reference genome should be supported by multiple sequences, such as transcript sequences and/or sequences from more than one BAC clone to avoid any possible sequencing errors (Additional file 4).

If none of the haplotypes of interest are sequenced, then reference genome sequence should be used to define the SNP maps of individual haplotypes by running the SHORE program. The SNP maps then can be compared to determine the SNPs between the haplotypes of interest. Once the candidate SNPs are identified, small PCR amplicons of ~ 200 bp can be amplified and digested with suitable restriction endonucleases to release the restriction length polymorphisms. A significant proportion of the SNPs could be unusable in SBP marker development because they may not be digested with restriction endonucleases in a haplotype- or genotype-specific manner. In such a case, one can apply derived CAPS (dCAPS) technology to improve the efficiency of SBP marker development [52].

Conclusions

A new molecular marker technology, based on genome sequence and physical map locations, is reported for those species whose assembled genome sequences are available. The technology was applied in identifying 21 SBP markers for some of the marker-poor genomic regions of the Arabidopsis molecular marker map that represent polymorphisms between ecotypes, Col-0 and Nd-0 (Figure 4). The SBP marker

technology should be applicable to any genomic regions and will facilitate (i) map-based cloning genes as well as (ii) the development of tightly linked molecular markers for selecting desirable genotypes in animal and plant breeding experiments.

Ease in SBP marker development and application to any genomic regions, and genome-wide abundance of SNPs make this technology suitable for mapping experiments, especially to develop high density molecular maps for positional gene cloning experiments, if the assembled genome sequence and physical maps of the studied species are available. Innumerable SBP markers can be developed rapidly for a genomic region containing a target gene in a map-based cloning experiment. Co-dominant gel-based SBP markers are ideal to identify genetic recombination events between two loci. Such PCR-based markers can be used to screen a large number of segregants to identify informative recombinants of the target gene region. These recombinants will then facilitate the development of high resolution maps of a large number of SBP markers, essential for cloning genes based on their map position. Thus, high-throughput deep sequencing, together with SBP markers, should expedite map-based cloning in higher eukaryotes.

Tables

Table 1 List of CAPS markers polymorphic between *Arabidopsis* ecotypes Col-0 and Nd-0

CAPS marker	Restriction enzyme	Primer Sequence
1H1L-1.6	<i>RsaI</i> , <i>Tsp509I</i>	F:CTAGAGCTTGAAAGTTGATG R:TTGAGTCCTTCTTGTCTG
20B4L-1.6	<i>DdeI</i>	F:CTAAGATGGGAATGTTGG R:GAACTCATTGTATGGACC
40E1T7	<i>AccI</i>	F:GGTCCACTTTGATTCAAGAT R:GCAAGCGATAGAACATAACG
AF2	<i>DdeI</i>	F:TCGTCGTTTTTGTTCCTTTTTCTTA R:CCATTCATTTAGGCCCGACTTTC
B9-1.8	<i>TaqI</i>	F:CATCTGCAACATCTTCCCCAG R:CGTATCCGCATTTCTTCACTGC
CAT2	<i>TaqI</i> , <i>Tsp509I</i>	F:GACCAGTAAGAGATCCAGATACTGCG R:CACAGTCATGCGACTCAAGACTTG
ER	<i>DdeI</i>	F:GAGTTTATTCTGTGCCAAGTCCCTG R:CTAATGTAGTGATCTGCGAGGTAATC
G4711	<i>DdeI</i>	F:CCTGTGAAAAACGACGTGCAGTTTC R:ACCAAATCTTCGTGGGGCTCAGCAG
GPA1.4	<i>Tsp509I</i>	F:ATTCCTTGGTCTCCATCATC R:GGGATTTGATGAAGGAGAAC
JM411	<i>DdeI</i>	F:GCGAACCCTAAGAACTA R:CTCGACTTTGCCAAGGAT
LFY3	<i>RsaI</i>	F:GACGGCGTCTAGAAGATTC R:TAACCTATCGGGCTTCTGC
MI342	<i>Tsp509I</i>	F:GAAGTACAGCGGCTCAAAAAGAAG R:TTGCTGCCATGTAATACCTAAGTG
M555	<i>AccI</i>	F:CCTTTAATTAGTTATCAAATC R:CTCTTGAATTATTAAGTTGACTAG
M59	<i>RsaI</i> , <i>Tsp509I</i>	F:GTGCATGATATTGATGTACGC R:GAATGACATGAACACTTACACC
MBK23A	<i>TaqI</i>	F:GATGATTAGGCGCAAAATTGAG R:ATTACCAGCCTGGCTTCAGG
PAI1.1	<i>TaqI</i> , <i>RsaI</i> , <i>Tsp509I</i>	F:GATCCTAAGGTATTGATATGATG R:GGTACAATTGATCTTCACTATAG
T20D161	<i>TaqI</i> , <i>RsaI</i> , <i>Tsp509I</i>	F:CGTATTTGCTGATTCATGAGC R:ATGGTTTACACTTGACAGAGC
T6P5-4.8	<i>RsaI</i>	F:TGAAAGACACCTGGGATAGGC R:CCAACCTTCGGGTCGGTTCC

Restriction endonucleases used for generating individual CAPS markers are shown. F, forward primer; R, reverse primer.

Restriction endonucleases used for generating individual SBP markers are shown. F, forward primer; R, reverse primer; C, Col-0; N, Nd-0.

Table 2 Primers and restriction enzymes used in generating 21 SBP markers

Ch. No.	Name	Primer sequence	Restriction enzyme	Size (bp)
1	SBP1_0.95	F:GTCAGGCTAGCTCATCAAGTCCTAC R:CCGGATTTTCGCCAGCTCCGTC	<i>Tsp509I</i>	100
1	SBP1_5.25	F:CACAAACCCTTCACCTCCAT R:GCAGTTGCCTAAAGGCTGAG	<i>MspI</i>	233
1	SBP1_8.08	F:AACGCAATTCTCAAGCAGGT R:CATTCAATTGTTGGGCAGTG	<i>RsaI</i>	188
1	SBP1_11.09	F:AAAGTCAACCGGGAGGTTTC R:AGGCTGAGGACACGAGAAGA	<i>BamHI</i>	163
1	SBP1_18.63	F:GCACTTGCAAAAGGAAGCTC R:TTCTTGCTGGAGAATCGTGA	<i>RsaI</i>	197
1	SBP1_22.30	F:TACCGGTTCCGGTCACTATC R:AATGGGAAATTGGGATTGGT	<i>DdeI</i>	151
1	SBP1_25.92	F:TTGTTGAGAGAGCGAGATCAAA R:AAAAGCATCACATCATCTTTGG	<i>NheI</i>	102
2	SBP2_14.07	F:GAAGGAATTGGACCAAACGA R:ATCTAGCTGCCCTCACTGGA	<i>BtsCI</i>	213
2	SBP2_16.23	F:CACCATTGTTCCTCGTAAGC R:TGGTCAATCCATGGTGATGT	<i>Hpy166II</i>	157
3	SBP3_6.60	F:CCATCGTCCTATTCTAATCCATGTTG R:GATGCAAAATCTCCATCCTCTTC	<i>Tsp509I</i>	379
3	SBP3_8.11	F:CACGTATCGGCGAGTCTACA R:CAAATTCAAATCTCAGTTTTTCGTC	<i>TaqI</i>	150
3	SBP3_10.28	F:TCTAAAACGAACCGGAAAA R:CGACAAGTAAATTAAAACCAACCTG	<i>MboII</i>	151
3	SBP3_11.12	F:AAGACTTTGGTTCAACTCCTGAA R:GGCTTTGGATTCAAGAAAAA	<i>TaqI</i>	184
3	SBP3_23.46	F:CGACCAAATGTCTCTGAGATGTTC R:CACCCAAGGCGGTGTTGGCGAAAG	<i>TaqI</i>	520
4	SBP4_0.67	F:CGGTTAACATGCCTCAATCC R:TGTGGATGATTTGGGGACTC	<i>DdeI</i>	171
4	SBP4_2.91	F:CGAGTGACTTCTTGAGGTTTATTATG R:CGAGATTGCTTTGGTATGGA	<i>Hpy166II</i>	249
4	SBP4_5.60	F:AGGGAAGAATATGCGGAAGG R:TGTTTCTGTCTTGCCCAT	<i>TaqI</i>	159
4	SBP4_6.51	F:GGACAAGACCTTGATTTGAAGTTTG R:GAGGGCTCACATTGGGTTTAATG	<i>Tsp509I</i>	395(C), 490(N)
5	SBP5_8.40	F:TCGACGGTGACTTGTAGGTG R:CGATGCCGTCTCATAAAAGG	<i>DpnI</i>	232
5	SBP5_14.60	F:CGCGGTTATGGTAACGTAAATG R:CCGAGGGAGAAGAAAGGATCAAGAAG	<i>HphI</i>	225
5	SBP5_25.00	F:AAATCACCAATGGCAAAACA R:TTTGCGTAGACGGAGAGTGA	<i>DdeI</i>	191

Restriction endonucleases used for generating individual SBP markers are shown. F, forward primer; R, reverse primer; C, Col-0; N, Nd-0.

Figures

Figure 1

Steps in generating SBP markers

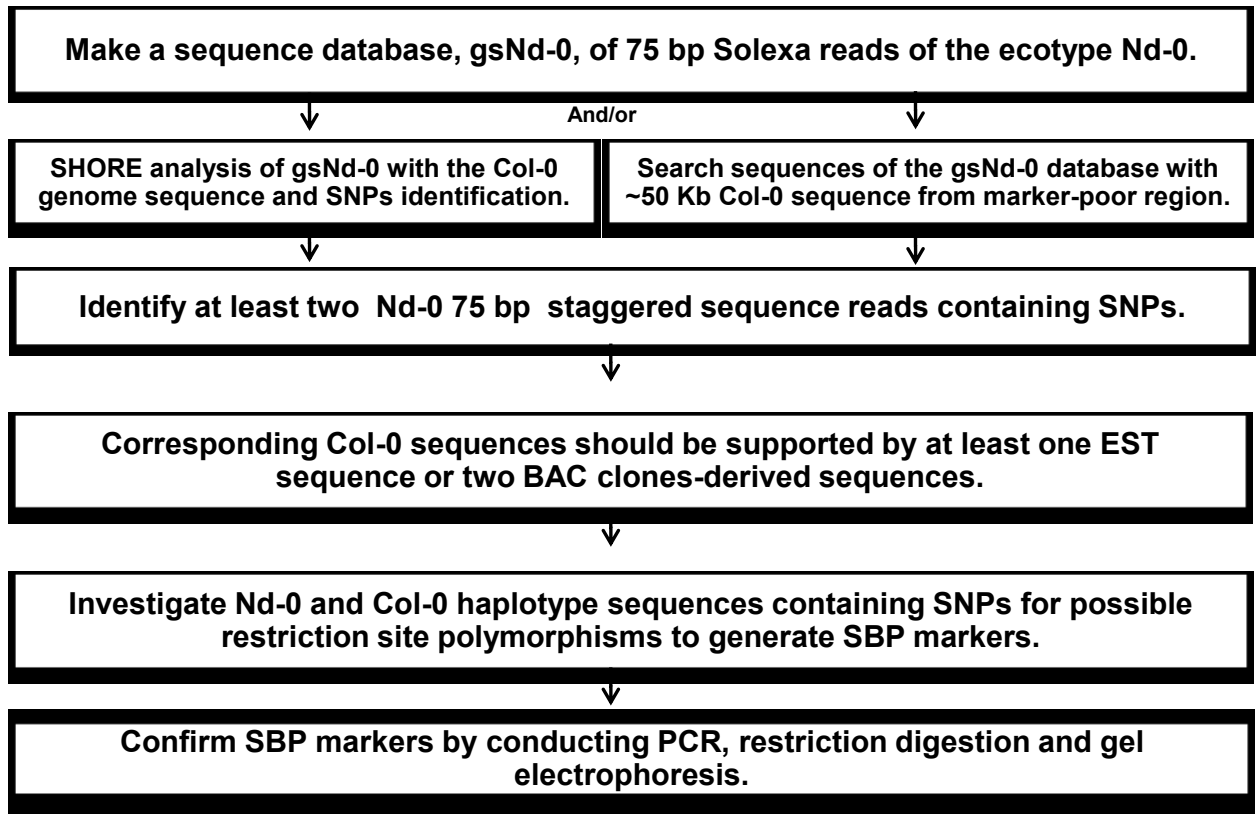


Figure 3

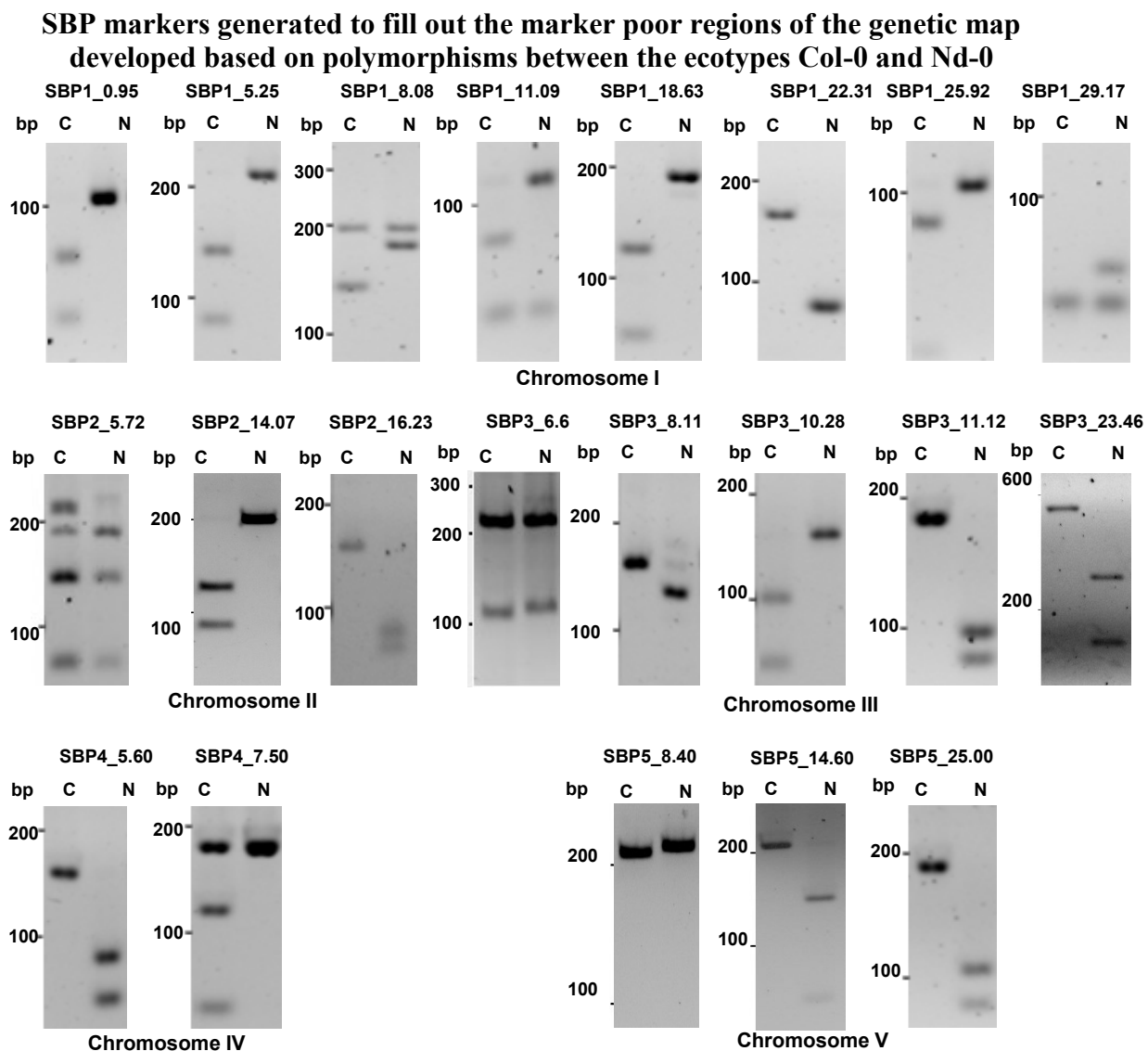
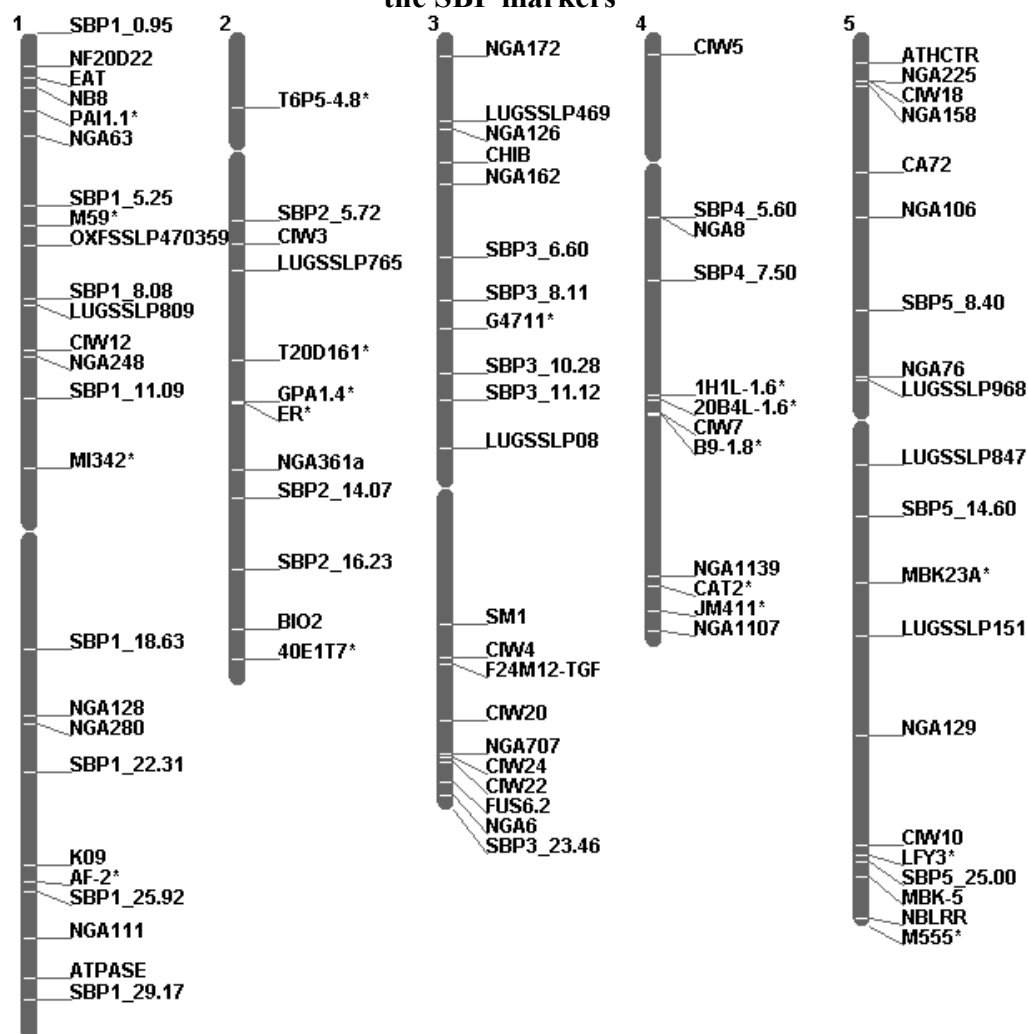
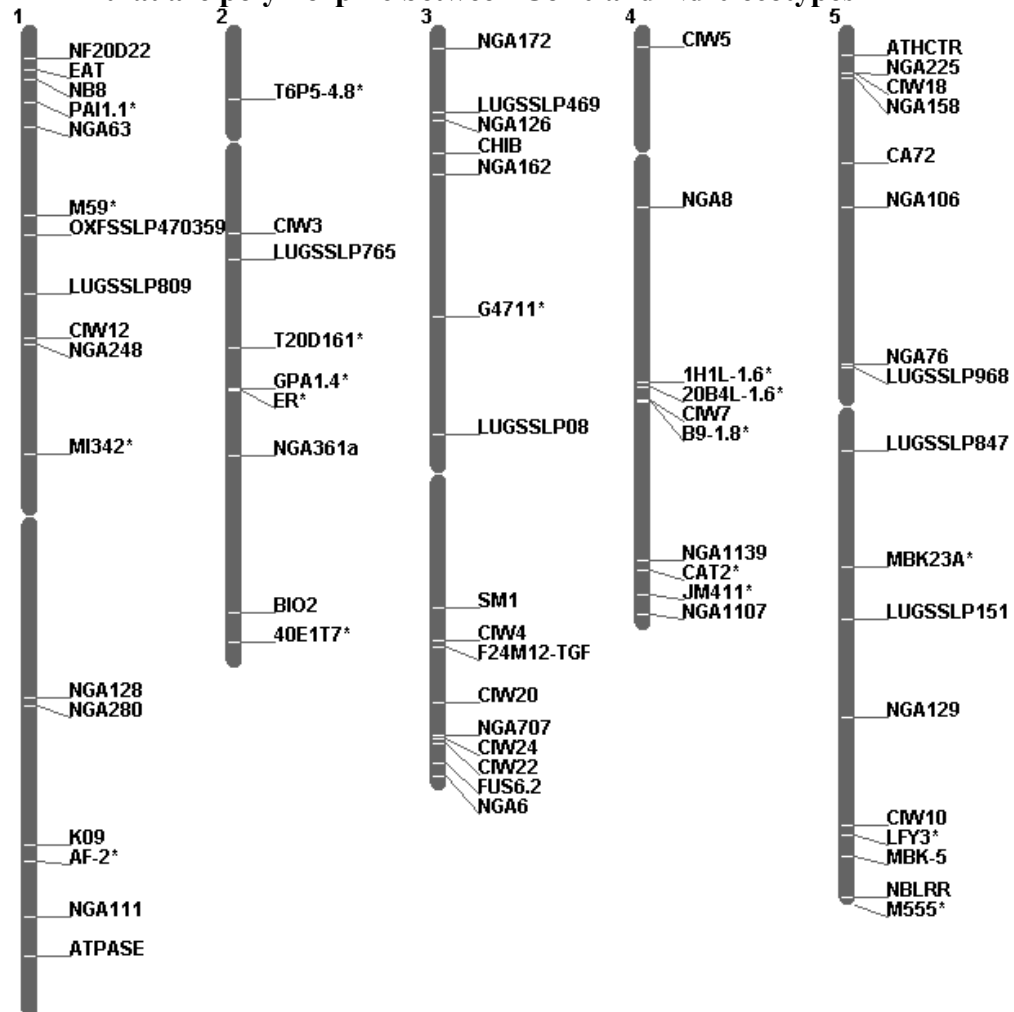


Figure 4

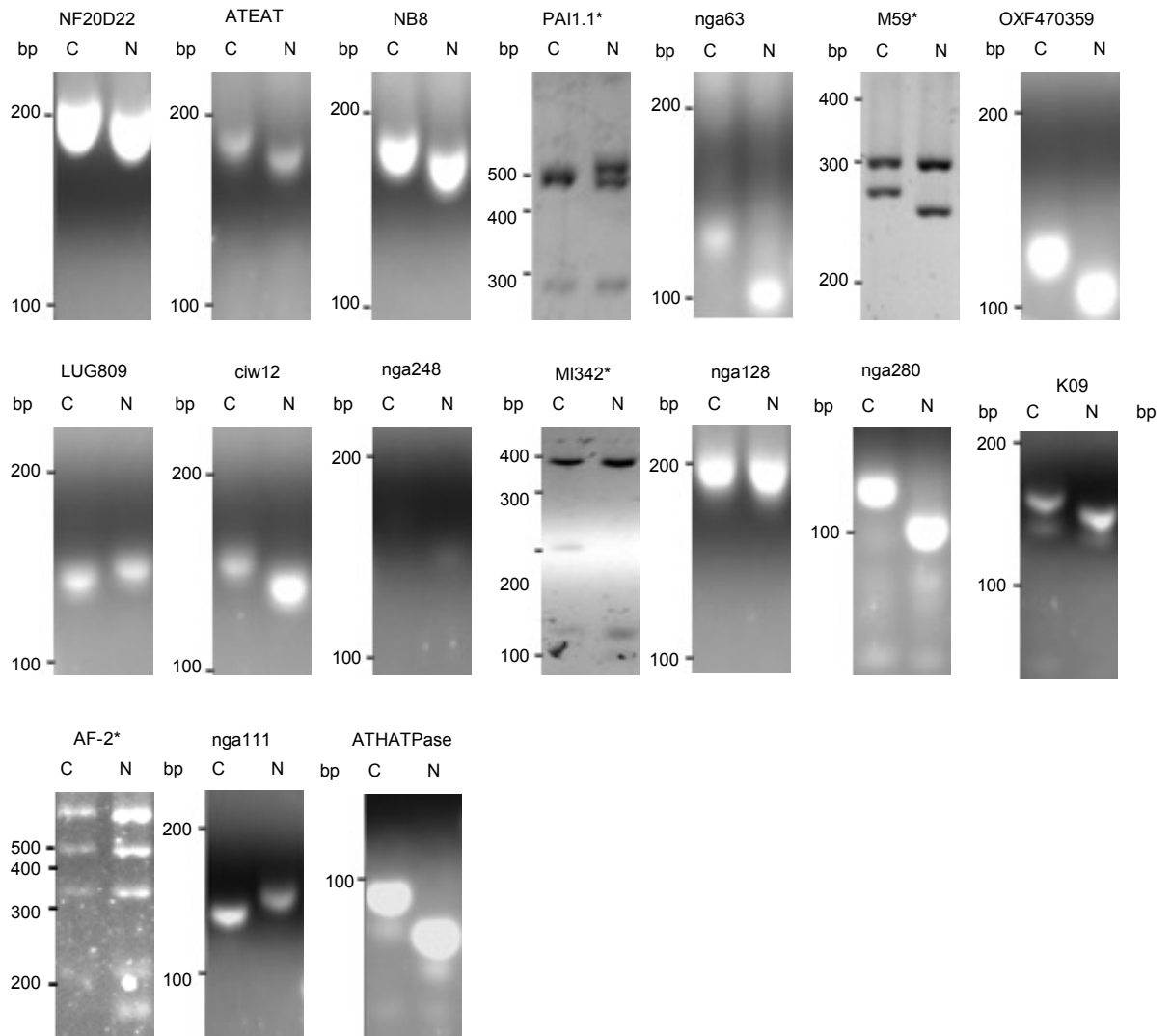
The molecular map of the five *Arabidopsis* chromosomes showing the locations of the SBP markers



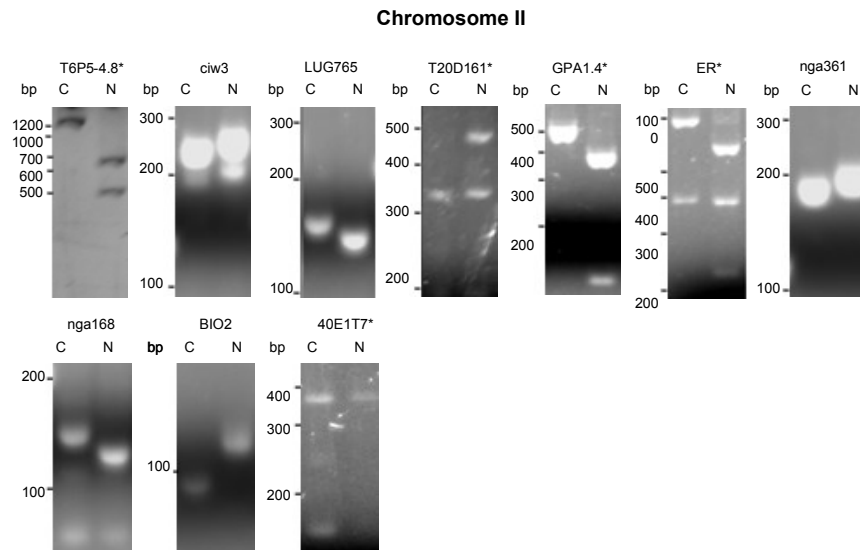
Arabidopsis molecular genome map generated based on SSLP and CAPS markers that are polymorphic between Col-0 and Nd-0 ecotypes



Additional File 2

Phenotypes of the SSLP and CAPS markers polymorphic between Col-0 and Nd-0 ecotypes**Chromosome I**

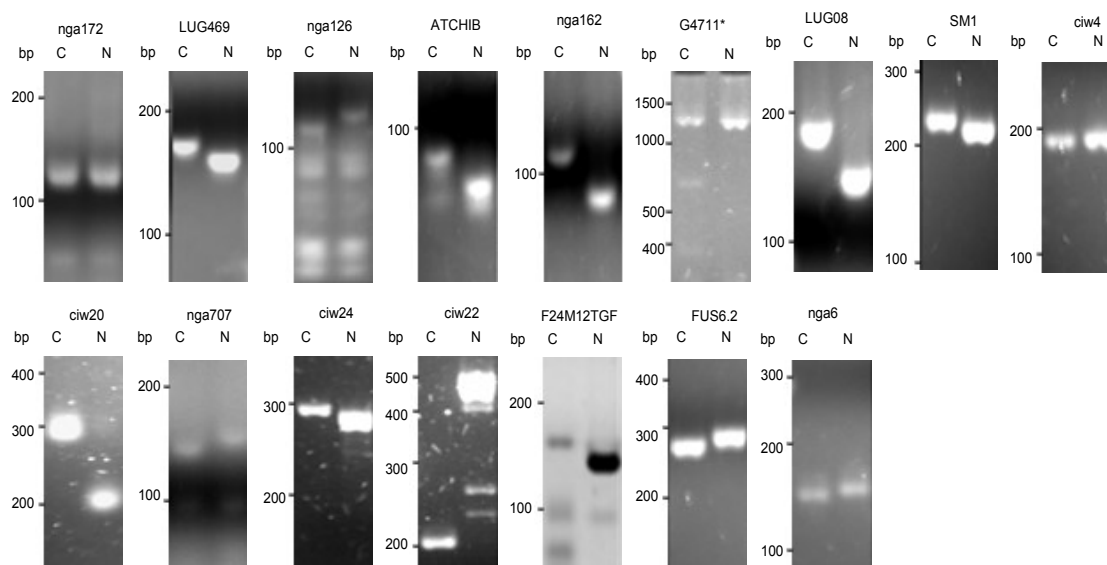
Additional File 2 continued

Phenotypes of the SSLP and CAPS markers polymorphic between Col-0 and Nd-0 ecotypes

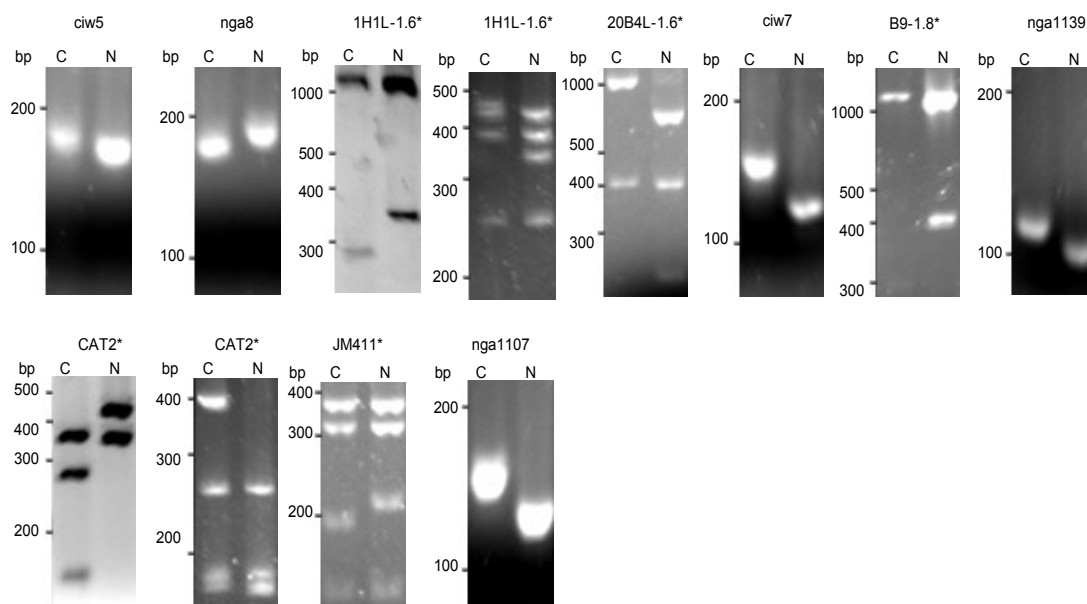
Additional File 2 continued

Phenotypes of the SSLP and CAPS markers polymorphic between Col-0 and Nd-0 ecotypes

Chromosome III

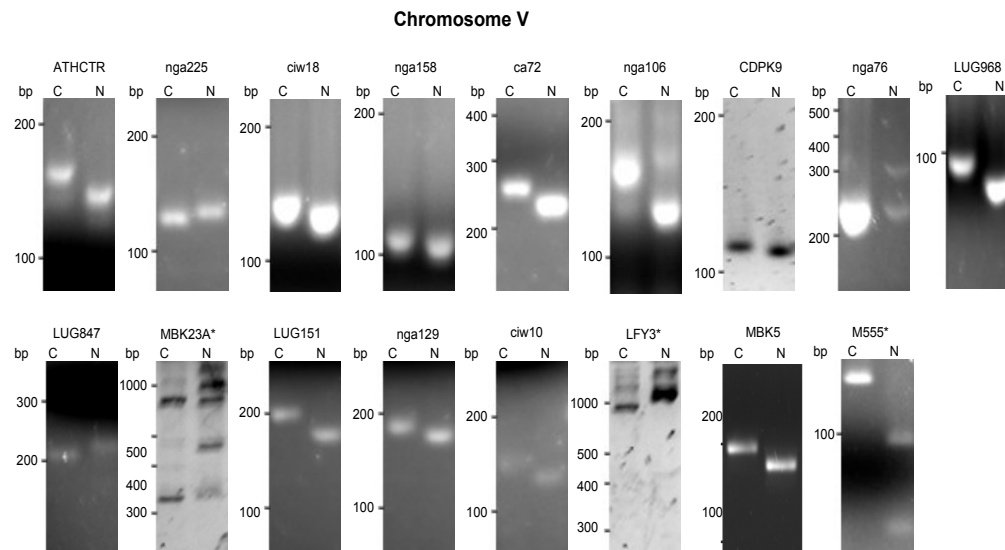


Additional File 2 continued

Phenotypes of the SSLP and CAPS markers polymorphic between Col-0 and Nd-0 ecotypes**Chromosome IV**

Additional File 2 continued

Phenotypes of the SSLP and CAPS markers polymorphic between Col-0 and Nd-0 ecotypes



Additional File 3

Two 75 bp Nd-0 Solexa reads most likely originated from a single DNA molecule

>HWI-EAS344:7:70:153:1969#0/1

Length = 75

Score = 137 bits (69), Expect = 4e-29

Identities = 69/69 (100%)

Strand = Plus / Plus

Query: 4863 atattatggtatcgcatgtatttcgaaaaataacatggtacaaaggacatttacgtaat 4922

|||||

Sbjct: 7 atattatggtatcgcatgtatttcgaaaaataacatggtacaaaggacatttacgtaat 66

>HWI-EAS344:7:17:1346:939#0/1

Length = 75

Score = 137 bits (69), Expect = 4e-29

Identities = 69/69 (100%)

Strand = Plus / Plus

Query: 4863 atattatggtatcgcatgtatttcgaaaaataacatggtacaaaggacatttacgtaat 4922

|||||

Sbjct: 7 atattatggtatcgcatgtatttcgaaaaataacatggtacaaaggacatttacgtaat 66

Additional File 4

The Col-0 sequence carrying SNPs, shown in Figure 2 (a), showed identity to three cDNA sequences

```
>[ref|NM_125629.2|] UEGM Arabidopsis thaliana invertase/pectin methylesterase inhibitor
family protein / DC 1.2-like protein (AT5G62350) mRNA, complete
cds
Length=937

GENE ID: 836356 AT5G62350 | invertase/pectin methylesterase inhibitor family
protein / DC 1.2 homolog (FL5-2I22) [Arabidopsis thaliana]
(10 or fewer PubMed links)

Score = 116 bits (60), Expect = 2e-25
Identities = 60/60 (100%), Gaps = 0/60 (0%)
Strand=Plus/Plus

Query 1 CTCACAGACCGAGCTTCCAAAAAGCCATAAACTTCATCCAATCTTCTTGCAAAACCAC 60
      |||
Sbjct 156 CTCACAGACCGAGCTTCCAAAAAGCCATAAACTTCATCCAATCTTCTTGCAAAACCAC 215

>[gb|AY072529.1|] UGM Arabidopsis thaliana ripening-related protein-like (MMI9.18)
mRNA, complete cds
Length=726

GENE ID: 836356 AT5G62350 | invertase/pectin methylesterase inhibitor family
protein / DC 1.2 homolog (FL5-2I22) [Arabidopsis thaliana]
(10 or fewer PubMed links)

Score = 116 bits (60), Expect = 2e-25
Identities = 60/60 (100%), Gaps = 0/60 (0%)
Strand=Plus/Plus

Query 1 CTCACAGACCGAGCTTCCAAAAAGCCATAAACTTCATCCAATCTTCTTGCAAAACCAC 60
      |||
Sbjct 78 CTCACAGACCGAGCTTCCAAAAAGCCATAAACTTCATCCAATCTTCTTGCAAAACCAC 137

>[emb|BX814277.1|] UM Arabidopsis thaliana Full-length cDNA Complete sequence from
clone GSLTFB65ZG09 of Flowers and buds of strain col-0 of Arabidopsis
thaliana (thale cress)
Length=752

Score = 116 bits (60), Expect = 2e-25
Identities = 60/60 (100%), Gaps = 0/60 (0%)
Strand=Plus/Minus

Query 1 CTCACAGACCGAGCTTCCAAAAAGCCATAAACTTCATCCAATCTTCTTGCAAAACCAC 60
      |||
Sbjct 602 CTCACAGACCGAGCTTCCAAAAAGCCATAAACTTCATCCAATCTTCTTGCAAAACCAC 543
```

Figure Descriptions

Figure 1 Steps in generating SBP markers. Putative SNPs were identified by (i) SHORE mapping of the 75 bp Nd-0 Solexa reads and Co-0 genome sequence and/or (ii) searching SNPs by comparing batches of 50 Kb Col-0 sequences with the 75 bp Nd-0 Solexa reads. The putative SNPs containing Solexa reads that carried staggered ends were selected for next step. Assembled genome sequences of Col-0 carrying putative SNPs were searched for 100% nucleotide matches with transcript sequences or sequences from multiple BACs. The SNPs were utilized to develop SBP markers if they could be translated to restriction fragment length polymorphisms.

Figure 2 Identification of SNPs for generation of SBP markers in *Arabidopsis thaliana*. (a) SHORE analysis of a 2.95 Kb DNA fragment of the lower arm of chromosome V between 25,034,700 and 25,037,650 bps resulted in four SNPs. Name, name of the project; Position, position within the chromosome; Ref base, nucleotide of the sequenced genome (Col-0); Cons base, Consensus base (Nd-0); Read type, part of the reads used for prediction were non-repetitive; Support, number of reads supporting a predicted feature; Concordance: Ratio of reads to total coverage of the sequenced genome. Max Quality, highest base quality supporting a prediction; Avg hits, average number of alignments of all reads covering this genomic position. (b) Two SNPs at positions 25,037,599 and 25,037,602 nucleotides [in bold font in (a)] were aligned in three Nd-0 Solexa reads with staggered ends. (c) Three 75 bp Nd-0 Solexa reads were aligned with the reference genome Col-0 (Query Sequence). Two SNPs were circled. Note that the three reads were from three independent DNA molecules.

Figure 3 SBP markers generated to fill out the marker poor regions of the genetic map developed based on polymorphisms between the ecotypes Col-0 and Nd-0. Primers for PCR amplification and restriction enzymes used in generating the SBP markers are listed in Table 2.

Figure 4 The molecular map of the five Arabidopsis chromosomes showing the locations of the SBP markers. Primers for 18 CAPS and 21 SBP markers are listed in Tables 1 and 2, respectively. Primer sequences for the 50 SSLP markers can be obtained from the TAIR database. CAPS markers are distinguished from SSLP markers with asterisks.

Additional files

Additional file 1: Arabidopsis molecular genome map generated based on SSLP and CAPS markers that are polymorphic between Col-0 and Nd-0 ecotypes.

CAPS markers shown with asterisks. The map was drawn using the chromosome map tool available at TAIR.

Additional file 2: Phenotypes of the SSLP and CAPS markers polymorphic between Col-0 and Nd-0 ecotypes.

C, Col-0; N, Nd-0.

Additional file 3: Two 75 bp Nd-0 Solexa reads most likely originated from a single DNA molecule.

The two reads showed similarity in their identity to a specific Col-0 sequence. Most likely the two sequence reads were obtained from sequencing of two molecules generated through PCR of a single DNA molecule.

Additional file 4: The Col-0 sequence carrying SNPs, shown in Figure 2 (a), showed identity to three cDNA sequences.

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CHAPTER 3. ARABIDOPSIS NONHOST RESISTANCE GENE *PSSI* CONFERS IMMUNITY AGAINST AN OOMYCETE AND A FUNGAL PATHOGEN BUT NOT A BACTERIAL PATHOGEN THAT CAUSE DISEASES IN SOYBEAN

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Abstract

Nonhost resistance (NHR) provides immunity to all members of a plant species against all isolates of a microorganism that is pathogenic to other plant species. Three *Arabidopsis thaliana* *PEN* (penetration deficient) genes, *PEN1*, 2 and 3 have been shown to provide NHR against the barley pathogen *Blumeria graminis* f. sp. *hordei* at the prehaustorial level. *Arabidopsis pen1-1* mutant lacking the *PEN1* gene is penetrated by the hemibiotrophic oomycete pathogen, *Phytophthora sojae*, that causes root and stem rot disease in soybean. We investigated if there is any novel nonhost resistance mechanism in *Arabidopsis* against the soybean pathogen, *P. sojae*.

The *P. sojae* susceptible (*pss*) 1 mutant was identified by screening a mutant population created in the *Arabidopsis pen1-1* mutant that lacks penetration resistance against the non adapted barley biotrophic fungal pathogen, *Blumeria graminis* f. sp. *hordei*. Segregation data suggested that *PEN1* is not epistatic to *PSSI*; therefore, *PSSI* must encode a new form of penetration resistance. The *pss1* mutant is also infected by the

necrotrophic fungal pathogen, *Fusarium virguliforme*, which causes sudden death syndrome in soybean. Thus, a common NHR mechanism is operative in *Arabidopsis* against both hemibiotrophic oomycetes and necrotrophic fungal pathogens that are pathogenic to soybean. However, *PSSI* does not play any role in immunity against the bacterial pathogen, *Pseudomonas syringae* pv. *glycinea*, that causes bacterial blight in soybean. We mapped *PSSI* to a region very close to the southern telomere of chromosome 3 that carries no known disease resistance genes.

The study revealed that *Arabidopsis PSSI* is a novel nonhost resistance gene that confers a new form of penetration resistance against both a hemibiotrophic oomycete pathogen, *P. sojae* and a necrotrophic fungal pathogen, *F. virguliforme* that cause diseases in soybean. However, this gene does not play any role in the immunity of *Arabidopsis* to the bacterial pathogen, *P. syringae* pv. *glycinea*, which causes bacterial blight in soybean. Identification and further characterization of the *PSSI* gene would provide further insights into a new form of nonhost resistance in *Arabidopsis*, which could be utilized in improving resistance of soybean to two serious pathogens.

Introduction

Plants are exposed to an innumerable number of pathogenic organisms on a daily basis. However, because of immunity mechanisms only a few pathogens can infect and cause diseases in a particular crop species. One of the less understood immunity mechanisms is nonhost resistance (NHR), exhibited by all members of a plant species against non- adapted pathogens [1-2]. The main NHR mechanisms were thought to be 1) incompatibility of non-adapted pathogen with the physiology of nonhost plants and 2) inability of non-adapted pathogens to overcome the plant defenses [3]. The first gene

known to confer *Arabidopsis* NHR against a non-adapted bacterial pathogen, *Pseudomonas syringae* pv. *phaseolicola*, is *NONHOST1* (*NHO1*) which encodes a glycerol kinase [4-5]. *NHO1* has also been shown to play an important role in the expression of gene-specific resistance against a bacterial pathogen [4].

NHR acts in two layers against the biotrophic fungal pathogens [6-7]. The first layer of NHR suppresses the invasion by non-adapted pathogens at the pre-haustorial level. Three NHR genes, *PEN1*, *PEN2* and *PEN3*, required for penetration resistance of *Arabidopsis* against the non-adapted barley biotrophic fungal pathogen, *Blumeria graminis* f. sp. *hordei* have been isolated [6-8]. These genes act at the prehaustorial stage of the pathogen invasion [9]. *PEN1* encodes a soluble N-ethylmaleimide sensitive attached receptor (SNARE) protein, which is involved in vesicle fusion and exocytosis of toxic compounds to the pathogen infection sites [8]. *PEN2* encodes a glycosyl hydrolase, which has been localized to the peroxisomes [6]. *PEN3* encodes an ATP-binding cassette (ABC) protein of the plasma membrane [7]. Cytological studies have demonstrated that *PEN2* and *PEN3* work together to generate and transport toxic chemicals into the infection sites [10]. The first layer of NHR prevents the biotrophic fungal pathogens from penetration and development of feeding structures, haustoria. Fungal pathogens that overcome the first layer of NHR encounter a post-haustorial defense mechanism. Some of the genes involved in the second layer of NHR in *Arabidopsis* are *EDS1*, *PAD4* and *SAG101* that are involved in plant defenses [6]. Downstream antagonistic defense pathways regulated by salicylic acid (SA) and the jasmonic acid (JA) are activated upon infection with biotrophic and necrotrophic pathogens, respectively [11]. SA and JA pathways are shown to be involved in the expression of nonhost resistance against the

cowpea rust, *Uromyces vignae*, in Arabidopsis [12]. Mutant studies have suggested that both SA and JA pathways are involved in nonhost resistance of Arabidopsis against the soybean pathogen *Phakopsora pachyrhizi* that causes the Asian soybean rust [13].

Recognition of pathogen associated molecular patterns (PAMPs) of non-adapted pathogens by PAMP recognition receptors (PRRs) triggers the PAMP-triggered immunity (PTI) in nonhost species [14]. Recent studies have shown PTI plays a major role in NHR [15]. Both chemical and physical barriers induced by PTI restrict non-adapted pathogens from invading nonhost species. Physical barriers include callose deposition at the infection sites and other preformed barriers such as waxy coating on leaves. Chemical barriers include deposition of various reactive oxygen species (ROS) such as hydrogen peroxide and phenolic compounds at the infection site [16-17].

The plant responses to pathogenic invasions can be classified into two broad groups, PTI and the effector-triggered immunity (ETI) activated by strain-specific effectors. Both PTI and ETI play roles in providing nonhost resistance of plant species against non-adaptive or nonhost pathogens. It is speculated that PTI and ETI play an increasingly major and a minor role, respectively, in conferring nonhost resistance as the evolutionary distance between the nonhost and the nonhost pathogen species widens [18]. Conversely, ETI and PTI play an increasingly major and a minor role, respectively, in expression of nonhost resistance as the evolutionary distance between the nonhost and nonhost pathogens reduces.

Soybean (*Glycine max* L. Merr.) is one of the most important oil seed crops, a major source of livestock feed and an important biodiesel crop. Unfortunately, soybean is also a host of many pathogens that cause several serious diseases resulting in an

estimated annual yield loss of \$2.26 billion dollars [19]. In the United States, the estimated annual soybean yield losses just from the oomycete pathogen, *P. sojae*, have been valued to be over 300 million dollars [19]. Although various *Rps* (resistance to *P. sojae*) genes are utilized in generating *Phytophthora* resistant soybean cultivars [20-21], resistance conferred by these genes is effective only against a set of *P. sojae* races and is not durable. Partial resistance governed by quantitative trait loci (QTLs) confers broad-spectrum resistance against *P. sojae* races in soybean. However, the level of partial resistance is not adequate enough to prevent significant crop losses [22]. Thus, it is essential to identify and use NHR mechanisms to provide soybean with broad-spectrum and durable resistance against this pathogen. As a first step towards achieving this goal, we have applied a forward genetic approach to identify and map the *Arabidopsis thaliana* NHR gene, *PSSI*, which provides resistance against the oomycete pathogen *P. sojae*. *PSSI* is also required for immunity of *Arabidopsis* against the fungal pathogen, *Fusarium virguliforme* that causes the sudden death syndrome (SDS) in soybean.

Material and Methods

Mutagenesis of *pen1-1*

About 15,000 *pen1-1* seeds were divided into three lots of ~5,000 seeds each. The three seed lots were then treated with 0.2%, 0.25%, and 0.3% EMS solution, respectively, for 15 h. The mutants were classified into three groups based on the concentration of EMS used for mutagenesis. Seeds were thoroughly washed 8 times in tap water and left in water on shaker for an additional hour. On an average, 1,000 seeds were sown on each flat (10-1/2" x 20-7/8"). Two weeks later plants were transplanted to trays containing 32

pots. The M_1 plants were selfed and seeds of 3,556 M_2 families were individually harvested.

Inoculation methods and disease scoring

Two methods of inoculation were applied: i) seedling inoculation and ii) detached leaf inoculation. For the seedling inoculation, more than 70 *A. thaliana* seeds of individual M_2 families were sterilized in the wells of 24-well microtiter plates (Costar[®] Corning Inc., Corning, NY) by first soaking in 70% ethanol for about 5 minutes and then washing with 50% Clorox bleach and 0.05% Triton X-100 for 10-15 minutes. The seeds were later rinsed four times with autoclaved water to remove any traces of bleach and/or ethanol. The seeds were then soaked aseptically in 300 μ l autoclaved, double distilled water and incubated at 4°C for 48 h followed by incubation at 22°C for 10 days under constant light (100 μ E/m²/s). Seedlings were then inoculated with 300 μ l *P. sojae* zoospores race 25 (10⁵ zoospores/ml). After two days of incubation at 22°C in the dark, the inoculated seedlings were stained with trypan blue and then destained with saturated chloral hydrate for 48 h [23]. Destained seedlings were mounted on a glass slide with glycerol and observed under a Zeiss microscope (Zeiss Incorporated, Thornwood, NY) and seedlings showing enhanced cell death in multiple cells were scored as susceptible. For the leaf inoculation, the seeds were sown on LC1 soil-less mixture (Sun Gro Horticulture, Bellevue, WA) under a 16 h light/8 h dark regime at 21°C with approximately 60% relative humidity. The light intensity was maintained at 120-150 μ E/m²/s [41]. Ten days after sowing, the seedlings were transplanted into a new LC1 mixture. The newly transplanted seedlings were covered with humidity domes for two

days and thereafter watered every fourth day. A fertilizer mixture of 15:15:15::N:P:K (1% concentration v/v) was applied to the seedlings seven days after transplantation.

Three leaves (leaf # 4, 5 and 6 from the apex) were detached from 21 day old plants and placed on moist Whatman filter papers, in Petri dishes. Each leaf was then inoculated with 10 μ l of *P. sojae* zoospore suspensions (10^5 /ml). The Petri dishes were covered and left under constant light ($50\mu\text{E}/\text{m}^2/\text{s}$) and at 22°C. The inoculated plants were scored 48 and 72 h post inoculation (hpi) for resistant and susceptible host responses. In some experiments, 10 μ l droplets of autoclaved, double distilled water were placed on the surface of detached leaves as a negative control.

Microscopic evaluations

Leaves of 21 day old Arabidopsis wild type Col-0 and the *pen1-1* and *pss1* mutant plants were inoculated with *P. sojae* spores (1.0×10^5 spores/ml) and stained with trypan blue 7 days post inoculation (dpi) [23] and with aniline blue dye at 6 hours post inoculation (hpi) [25]. The stained leaves were mounted in saturated chloral hydrate for trypan blue dye [23] or in 70% glycerol and 30% aniline blue solution (0.01%) for aniline blue dye [25]. Stained images were examined using a Zeiss Axioplan II compound microscope equipped with AxioCam color digital camera.

DNA preparation, PCR and BSA

Arabidopsis genomic DNA was extracted by CTAB method [42]. Young inflorescence or a rosette leaf was selected for DNA extraction. Equal amount (10 μ g) of DNA from individual $F_{2:3}$ families were mixed to obtain bulk DNA samples. The final DNA concentration of these bulk DNA samples for PCR was 20 ng/ μ l. The PCR reaction mixtures contained 2 mM MgCl_2 (Bioline, Taunton, MA), 0.25 μ M each of forward and

reverse primer (Integrated DNA Technologies, Inc., Coralville, Iowa), 2 μ M dNTPs and 0.5 U Choice Taq polymerase (Denville Scientific, Inc., Metuchen, NJ). For SSLP markers, PCR was conducted at 94°C for 2 min, and then 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. Finally, the mixture was incubated at 72°C for 10 min. For the CAPS markers, PCR was conducted at 94°C for 2 min, and then five cycles of 94°C for 30 s followed by decreasing annealing temperatures from 55°C to 50°C (-1°C/cycle) and 72°C for 1 min. Then 40 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min were conducted. Finally, the reaction mixture was incubated at 72°C for 10 minutes. PCR was carried out in PTC-100 Programmable Thermal Controllers (MJ Research Inc.). The amplified products were resolved on a 4% agarose gel by running at 8 V/cm. The ethidium bromide stained PCR products were visualized following illumination with UV light.

RNA isolation and RT-PCR experiments

Total RNA was isolated from leaf tissues using TRIzol ® reagent according to the manufacturer's instructions (Invitrogen, Inc., Carlsbad, CA).. RNA samples were treated with DNaseI (Invitrogen, Inc., Carlsbad, CA) to eliminate any DNA contamination [43]. cDNAs were prepared according to manufacturer's recommendations (Invitrogen, Inc., Carlsbad, CA). *PsAvh223*, *PsAvh224* and *AtACTIN* specific primers (Additional file 5) were used to PCR amplify cDNA fragments from these samples. RT-PCR was conducted for the above genes using the cDNAs prepared from infected leaves at 1 d and 3 d post inoculation or treatment with water droplets. The following program was used to conduct PCR; 94°C 3 min and 35 cycles of 94°C for 30 sec, 60°C or 55°C and 72°C for 1 min followed by 72°C for 10 min. The transcripts of *AtACTIN* were simultaneously amplified

for each set of RT-PCR reaction to show the possible variations in starting RNA amounts of different samples.

Molecular markers

Primers for SSLP markers were obtained from The Arabidopsis Information Resource (TAIR) database (<http://www.arabidopsis.org>). Candidate SSLP markers were selected to cover the entire genome with a density of one SSLP marker/2 Mb DNA. For the SSLP thin regions, CAPS and SBP markers were designed [34]. The primers for the CAPS are presented in Table 3 and that for the SBP markers are presented in Table 4.

Seedling inoculation with *F. virguliforme*

For inoculation of F_{2:3} families with *F. virguliforme*, more than 70 seedlings of each family were grown in 24-well microtiter plates (Costar[®] Corning Inc., Corning, NY) as described earlier. The seedlings of individual family were then inoculated with about 300 µl *F. virguliforme* spores (10⁶ spores/ml) and incubated in the dark for 48 h. The inoculated seedlings were then stained with trypan blue dye as previously described and observed under a microscope (Zeiss Inc., Thornwood, NY). Seedlings showing enhanced cell death in multiple cells were scored as susceptible.

Leaf inoculation of RILs with the bacterial pathogen, *P. syringae* pv. *glycinea*

For leaf inoculation of RILs with *P. syringae* pv. *glycinea*, Arabidopsis plants were grown in a 10 h light/14 h dark period at 21°C under light intensity of 100-120 µmol/cm²/sec. *P. syringae* pv. *glycinea* was grown on King's B medium containing rifampicin (100 µg/ml) at 28° C. For liquid culture, bacteria were grown in liquid King's B medium without rifampicin at 25°C for 24 h. Four-week old plants were leaf inoculated

with bacterial suspensions with 0.10 OD_{600nm} ($\sim 2 \times 10^6$ cfu/ml) diluted in 10mM MgCl₂ solution [44]. Four leaves of each plant were inoculated on the abaxial side with 50 μ l bacterial suspensions using the blunt end of a 1ml syringe (BD, Franklin Lakes, NJ). Plants were then covered with a humidity dome until samples were harvested for plating. 1 cm diameter leaf discs from each inoculated leaf samples were harvested at 0 and 3 days post-inoculation. Leaf discs of eight leaves from two plants were pooled to make one replication and three biological replications were performed. Serial dilutions of the extracts from leaf disc samples were plated on King's B medium containing rifampicin. Colony forming units (cfu) were counted 2 days following plating.

Results

Arabidopsis pen1-1* mutant, but not *nho1* mutant, is penetrated to single cells by the soybean pathogen *P. sojae

Arabidopsis nho1 and *pen1-1* mutants are defective in NHR mechanisms against the bacterial pathogen, *Pseudomonas syringae* pv. *phaseolicola* [5] and the powdery mildew fungus, *Blumeria graminis* f. sp. *hordei* [8], respectively. We investigated if the soybean pathogen *P. sojae* infects either of the two mutants. Ten day old seedlings grown in autoclaved double distilled water were inoculated with *P. sojae* zoospore suspensions and incubated for three days in the dark at 22°C. The inoculated seedlings were then stained with trypan blue dye and observed under a light microscope [23]. The pathogen did not penetrate either the wild-type ecotype Columbia-0 (Col-0) or the *nho1* mutant (Figures 1A and B). *P. sojae* however penetrated single cells in *pen1-1* (Figure 1C). These results indicated that in the *pen1-1* mutant the pre-haustorial NHR against *P. sojae* is compromised.

Identification of *Phytophthora sojae* susceptible (*pss*) putative mutants

We mutagenized *pen1-1*, compromised in pre-invasive immunity against *P. sojae*, with ethyl methane sulfonate (EMS) to identify mutants that are compromised in post-invasive immunity mechanisms. Over 3,500 M₁ plants were planted and M₂ seeds of these plants were harvested individually. Three hundred and seventy-nine randomly selected M₂ families were grown to score for the chlorophyll mutants, a marker for determining the extent of EMS-induced mutation. About 5% of the families segregated for albino plants (Additional file 1), which suggested that the mutant population contained sufficient random point mutations and suitable for screening. Approximately ≥ 70 seedlings of each M₂ family were grown aseptically in 24-well microtiter plates in sterile water at 22°C for 10 days before inoculating with *P. sojae* zoospores. Following inoculation, seedlings were incubated for two days at 22°C in the dark, and then seedlings were stained with trypan blue for identifying putative mutants via staining of dead infected cells [23]. From screening 3,500 M₂ families, we identified 30 putative mutants that were penetrated by *P. sojae* to multiple cells. The putative mutants were named as *Phytophthora sojae* susceptible 1 (*pss1*) through *pss30*. Subsequently, a detached leaf inoculation technique, previously reported for soybean, was applied in screening the putative mutants to identify the homozygous mutant plants [24]. We have applied a mapping approach in classifying these putative mutants. A homozygous mutant M₄ family (0.2B₁₇I₉-24) of the putative mutant *pss1* showing complete loss of both pre- and post-haustorial NHR against *P. sojae* was selected. In successive generations, the selected *pss1* mutant family was consistently infected by *P. sojae*. This mutant was phenotypically different from the *pen1-1* because death in the mutant seedlings occurs in

multiple cells as compared to in single cells in the *pen1-1* mutant (Figures 1D, E, F, G, H). Although the *P. sojae* zoospores germinated and were able to form appresoria at the infection site, its growth was arrested immediately following germination in wild type Col-0 leaves. The *pen1-1* mutant showed occasional death in single cells following *P. sojae* infection. Microscopic evaluations showed distinct phenotypic differences among wild-type Col-0, *pen1-1* and *pss1* mutants following infection with *P. sojae*.

To determine the extent of *P. sojae* growth in infected tissues, detached *pss1* leaves were collected 6 hours post inoculation (hpi) with zoospore suspensions or treatments with water droplets. Leaves were then stained with aniline blue and the ultraviolet epifluorescence was visualized using a Zeiss Axioplan II compound microscope [25]. Extensive colonization by the pathogen was observed in the *pss1* mutant (Figure 2A). Aniline blue stains the callose deposition and papillae formation and can be used to visualize fungal structures such as runner hyphae [26-27]. Callose deposition and papillae formation has previously been used as a marker for attempted penetration sites by fungal pathogen [7]. Following inoculation with *P. sojae* zoospores, *pss1* leaves showed extensive callose deposition and papillae formation across the infected leaf tissue as compared to *pen1-1* and Col-0 (Figure 2A). Neither callose deposition nor papillae formation was detected in detached leaves that were treated with water droplets (Additional file 2A). At 6hpi, extensive growth of the secondary hyphae was observed in *P. sojae* infected leaves of *pss1* but not that of Col-0 and *pen1-1* (Figure 2A).

To determine if *P. sojae* became adapted to the Arabidopsis *pss1* mutant, we conducted microscopic study of the diseased lesions of the detached *pss1* leaves 7 days post-inoculation (dpi) with the zoospore suspensions of the oomycete (Figure 2B). We

observed enhanced hyphal growth and formation of reproductive structures, sporangia and oogonia on *pss1* leaves (Figure 2B, Additional file 2B). Thus, we conclude that a gene mutated in *pss1* is crucial for nonhost immunity of Arabidopsis against the soybean pathogen, *P. sojae*. We named this gene *PSSI*.

Arabidopsis ecotypes showed leakiness in their NHR responses to *P. sojae*

Columbia-0 (Col-0) and *Landsberg erecta* (Ler) are the two most well characterized ecotypes of *Arabidopsis thaliana* for mapping and gene cloning experiments [28-29]. We investigated if the ecotype Ler was completely immune to *P. sojae* so that it could be crossed to *pss1* for generating mapping populations. However, Ler showed leakiness in its immune response against *P. sojae* and a significant proportion (12.5%) of the Ler seedlings were infected by *P. sojae* (Table 2). This result is not very surprising because the Arabidopsis ecotype *L. erecta* has recently been found to show susceptibility to another oomycete pathogen, *Pythium irregulare* [26]. We therefore inoculated 22 *A. thaliana* ecotypes with *P. sojae* zoospores and discovered that ecotypes, Bensheim, Nossen-0 (No-0) and Niederzenz-0 (Nd-0) were completely immune to the pathogen (Table 2). We selected Nd-0 for mapping experiments because it is morphologically similar to Col-0. Furthermore, a few molecular markers polymorphic between Nd-0 and Col-0 were already available [30].

PSSI* is required for nonhost resistance of Arabidopsis against *P. sojae

Forty-two F_{2.3} families developed from the cross between *pss1* and Nd-0 were evaluated for segregation of host responses to the pathogen infection. At least 24 progenies of each F₂ plants were scored for disease phenotypes. The segregation of alleles at the *PSSI* locus among the F_{2.3} families fit to the 1:2:1 genotypic ratio for a

single gene model ($p = 0.81$; Table 1). This observation suggested that *PSSI* is a single gene with no apparent epistatic effect from *PEN1*.

In addition to these 42 $F_{2:3}$ families, we determined the phenotypes of additional families. In this experiment, only eight progenies per family were screened to identify the $F_{2:3}$ families that carry *pss1* in homozygous condition. To further confirm that *PSSI* is a single gene with no epistatic effect from *PEN1*, we evaluated the segregation of the *PEN1* alleles among 20 $F_{2:3}$ families, homozygous for the *pss1* allele, using the dCAPS marker for *PEN1* alleles [7]. *PEN1* alleles segregated in a 1:2:1 ratio ($p = 0.67$) among the 20 families, homozygous for the *pss1* allele (Figure 3). This result suggested an independent segregation for the two genes. Among the 20 homozygous families for the *pss1* allele, four showed to carry the *PEN1* allele in homozygous condition. If the *PEN1* allele was epistatic to *PSSI* and *PSSI* were to encode only a post-invasive resistance mechanism, then the *pen1-1* allele should have been in recessive homozygous condition among the *pss1* homozygous families. Thus, *PSSI* encodes a new form of penetration resistance. The new mutation was therefore named *pss1* instead of *pen1-1pss1* because *pen1-1* mutation played no role in development of the *pss1* phenotype.

Expression of *P. sojae* effector genes in *pss1* during infection

To determine the extent of *P. sojae*-gene expression, we selected two effector genes to conduct RT-PCR. It has been shown that *P. sojae* carries over 370 candidate effector proteins containing N-terminal RXLR-dEER motifs [31]. We studied the expression of *PsAvh223* and *PsAvh224* [32] in *pss1*, *pen1-1* and Col-0 following inoculation with *P. sojae*. Both effector *P. sojae* genes were highly expressed in the *pss1*

mutant as compared to *pen1-1* and Col-0 (Figure 4). This result indicates that the *P. sojae* colonized to a greater extent in *pss1* as compared that in *pen1-1* or Col-0.

Mapping of the *PSSI* gene

In order to map the *PSSI* gene, we applied bulked segregant analysis (BSA) [33]. Four bulks of *P. sojae* susceptible plants each carrying 7-8 F_{2:3} susceptible families and one bulk of *P. sojae* resistant plants containing two homozygous (*PSSI**PSSI*) and six heterozygous (*PSSI**pss1*) F_{2:3} families were generated. These five bulks and Col-0 and Nd-0 were included in BSA. We used sequence-based polymorphic (SBP) [34] , SSCP and CAPS markers in conducting BSA.

The *PSSI* region was putatively mapped to the south arm of chromosome 3 (Figure 5A). To develop a high density map of the *PSSI* region, five SBP markers from this region were generated. SBP_20.71 marker showed a recombination event with the *PSSI* locus in the F_{2:3} family 93 suggesting that *PSSI* is located south of this marker (Figure 5B). No recombination was observed between *PSSI* and SBP_23.46 marker, located at the telomeric end of chromosome 3 (Figure 4C). The physical distance between SBP_20.71 and SBP_23.46 is ~2.75 Mb.

The Arabidopsis *pss1* mutant is infected by the fungal pathogen, *Fusarium virguliforme*, which causes sudden death syndrome in soybean

We investigated if *PSSI* controls Arabidopsis NHR against the fungal pathogen, *F. virguliforme* that causes sudden death syndrome (SDS) in soybean. From the segregating materials used for mapping the *PSSI* gene, we identified six F_{2:3} families that were homozygous for either *PSSI* or *pss1* alleles (Additional file 3) and used these families in determining the role of *PSSI* in NHR of Arabidopsis against *F. virguliforme*.

Seedlings of the selected families were grown in 24-well microtiter plates for 10 days and then inoculated with *F. virguliforme* conidial spores. Infected seedlings were stained with trypan blue and observed under a light microscope (Figure 6A). Significant proportions of seedlings in six families carrying only the *pss1* allele were infected by the fungal pathogen (Figure 6B). This result suggests that *PSSI* is also essential for NHR against the soybean pathogen, *F. virguliforme*.

***PSSI* is not required for NHR of Arabidopsis against the non-adaptive pathogen *Pseudomonas syringae* pv. *glycinea* that causes bacterial blight in soybean**

We investigated if *PSSI* is required for NHR of Arabidopsis against the bacterial pathogen, *Pseudomonas syringae* pv. *glycinea* (*Psg*) that causes bacterial blight in soybean [35]. We inoculated the six F_{2:3} families that were homozygous for *pss1* and five F_{2:3} families that were homozygous for the *PSSI* allele with *Psg* (Figure 6C). We observed no association of *PSSI* and *pss1* alleles with the colony forming units (cfu) of the bacterial pathogen. We classified the responses of the selected families into two broad groups, one with cfu comparable to those observed for Col-0 and Nd-0; and the other one with five- or more-fold lesser cfu as compared to those observed in Col-0 and Nd-0. Surprisingly, *pen1-1* consistently showed about 4-5-fold less bacterial growth as compared to that in Col-0 (Figure 6C). To determine if *PEN1* is required for growth of *Psg*, we genotyped the selected susceptible and resistant F_{2:3} families for the *PEN1* locus (Additional file 4). No association was observed between alleles at the *PEN1* locus and the levels of *Psg* cfu. These results suggested that an unknown mutation in the *pen1-1* genotype is most likely involved in enhancing resistance of Arabidopsis against *Psg* (Figure 6C) and the unknown gene could be a negative regulator of disease resistance.

Discussion

Arabidopsis nonhost gene *PSSI* is a novel nonhost resistance gene which confers immunity of Arabidopsis against two non-adaptive soybean pathogens, *P. sojae* and *F. virguliforme*

Transfer of NHR mechanisms across species may lead to development of broad-spectrum and durable resistance in economically important crop species. Identification of *NHO1* and *PEN* genes established the molecular basis of NHR. It also suggested the feasibility of transferring single gene-encoded NHR across plant species for creating durable and broad-spectrum resistance [4, 6-8].

Here we have described a new Arabidopsis locus *PSSI* as one of the genes that provides nonhost immunity against two important soybean pathogens, *P. sojae* and *F. virguliforme*. Considering the disease phenotypes observed in detached leaves of *pss1* as opposed to that in detached leaves of the *pen1-1* mutant following *P. sojae* inoculation (Figures 1 and 2), the NHR mechanism governed by *PSSI* is most likely important not only to provide penetration resistance, but also to confer necessary protection against further spread of the pathogen. *pss1* supports secondary hyphal growth of *P. sojae* (Figure 2). Based on these observations we hypothesize that *PSSI* encodes a NHR defense mechanism that regulates both penetration and post-penetration resistance. It has been shown that the post-haustorial stage of the NHR mechanism is most important in sow thistle for providing resistance against a poorly adapted powdery mildew fungus, *Golovinomyces cichoracearum* UMSG1 [36]. Similar mechanism could also be important for NHR of Arabidopsis against the non- adapted oomycete pathogen, *P. sojae*.

Segregation data from a cross between *pssI* and Nd-0 revealed 1:2:1 genotypic segregation ratio for the alleles at the *PSSI* locus (Table 1); and therefore, it is a single gene. Alleles at the *PEN1* locus segregated independently of the alleles at the *PSSI* locus (Figure 3). The *P. sojae* susceptible phenotype of the *pssI* allele is manifested even in the presence of *PEN1* in the homozygous condition. Thus, *PSSI* controls a novel defense mechanism for penetration resistance against the oomycete pathogen, *P. sojae* and the fungal pathogen, *F. virguliforme*. *PEN* genes have been shown to regulate two distinct NHR mechanisms that are involved in penetration resistance. Monogenic inheritance of *PSSI* with no epistatic effect from *PEN1* suggests that an additional Arabidopsis NHR mechanism is operative against penetration by oomycete and *Fusarium* pathogens. *PSSI* is located in an approximately 2.75 Mb region flanked by two sequence-based polymorphic markers, SBP_20.71 and the telomere-specific SBP_23.46 (Figure 4C). This region does not contain any characterized plant defense or disease resistance genes. Thus, most likely we have identified a novel nonhost resistance mechanism in Arabidopsis.

The important hallmarks of a successful adapted pathogen are its ability to establish feeding structures, derive nutrition from the host and finally to complete its lifecycle in the host plant [3]. Aniline blue staining has previously been used to show oomycete feeding structures such as runner hyphae [26]. We observed secondary hyphae even after 6 hpi suggesting that *P. sojae* is able to form feeding structures in *pssI* leaves at a very early stage following inoculation (Figure 2A). Sporangia are specialized asexual reproductive structures of oomycetes which can either germinate into hyphae or release about 10-30 zoospores to complete the asexual life-cycle under. The male and female reproductive structures, antheridia and oogonia, are fused to develop oospores and

complete the sexual life [37]. *P. sojae* developed both sporangia and oogonia in infected *pss1* leaves; and thus, completed its life cycle in this mutant (Figure 2B). In contrast, in *pen1-1* leaves the pathogen was able to penetrate single cells, which was however subsequently followed by host cell death; while in the wild type Col-0 leaves, germinated *P. sojae* zoospores failed to penetrate host cells (Figure 2B).

***PSS1* encodes a novel NHR mechanism that regulates both pre- and post-invasive resistance of Arabidopsis against the nonhost pathogen**

Lack of epistasis of *PEN1* on *PSS1* (Figure 3), growth of secondary hyphae and rapid induction of effector genes in the *pss1* mutant, and most importantly completion of the *P. sojae*'s life cycle in infected *pss1* mutant leaves suggest that *PSS1* encodes a novel NHR mechanism that regulates both pre- and post-invasive resistance of Arabidopsis against the nonhost pathogen. Transfer of this to soybean could play an important role in creating broad-spectrum disease resistant not only against *P. sojae*, but also *F. virguliforme*. It is also possible that *PSS1* encoded resistance may be applicable to fighting diseases caused by oomycete pathogens in other crop species; such as potatoes, tomatoes, etc.

It has been shown that lack of either of a functional pathway, the *PEN1*/*SNAP33*/*VAMP721/722* or the indole- glucosinolates/metabolites pathway, involving the *PEN2*/*PEN3* activity is sufficient to allow a non-adapted fungal pathogen to enter Arabidopsis mutant plants at a rate similar to that in an adapted host [38]. However, a complete loss of the subsequent post-invasion resistance mechanism encoded by plant defense genes *PAD4* and *SAG101* is necessary for a nonhost plant species to become a host for such non-adapted fungal pathogens [18]. In light of the critical role of the post-

invasion genes as determinants of the nonhost status of *Arabidopsis* against non-adapted fungal pathogens, *PSSI*'s role at both pre- and post-haustorial levels in conferring NHR of *Arabidopsis* against *P. sojae* is novel.

Transfer of this to soybean could play an important role in creating broad-spectrum disease resistant not only against *P. sojae*, but also *F. virguliforme*

In vivo trans-specific gene silencing in *Fusarium verticillioides* from transgenic tobacco provides molecular evidence suggesting a possible short biotrophic phase in *Fusarium species* [39]. *F. virguliforme* has been considered to be semi-biotrophic fungus with its ability to feed on live host soybean cells [40]. Thus, most likely *PSSI* may regulate the immunity against both hemibiotrophs, *P. sojae* and *F. virguliforme*, by using the same mechanism. The differing lifestyles of the two pathogens, *P. sojae* and *F. virguliforme* and the importance of *PSSI* in providing nonhost resistance against both these pathogens hints at a crucial role of this gene in broader nonhost resistance of the model plant, *Arabidopsis*. Further studies would be required to characterize the role of *PSSI* in terms of the interplay of SA and JA- mediated defense pathways in response to infection by biotrophic and necrotrophic pathogens and its precise role in the complex mechanism of nonhost resistance.

Analyses of the segregants homozygous for alleles at both *PEN1* and *PSSI* loci revealed that *PEN1* does not have any epistatic effect on *PSSI* function. The present study thus revealed a novel nonhost gene, *PSSI*, which confers immunity of *Arabidopsis* against two non-adaptive soybean pathogens, *P. sojae* and *F. virguliforme*. Responses of *pss1* and *pen1-1* to *P. sojae* invasion were distinct and suggest that *PSSI* may act at both pre- and post-haustorial levels, while *PEN1* acts at the pre-haustorial level. Identification

and further characterization of the gene would provide us further insights about this new form of nonhost resistance against two non-adaptive soybean pathogens. This study thus laid the foundation for possible development of soybean germplasm with durable resistance against two serious pathogens.

Tables

Table 1 Segregation of *PSSI* alleles among the F_{2:3} families derived from a cross between the *pssI* mutant and the ecotype Nd-0.

Genotype	Observed	Expected
Homozygous resistant (<i>PSSI</i> <i>PSSI</i>)	12	10.5
Heterozygous (<i>PSSI</i> <i>pssI</i>)	21	21
Homozygous susceptible (<i>pssI</i> <i>pssI</i>)	9	10.5
Total	42	42
χ^2 value	0.43	
<i>P</i> -value	0.81	

Table 2 Responses of Arabidopsis ecotypes to *P. sojae*.

Ecotypes	Seedling Inoculation			Leaf Inoculation		
	¹ Immune	² Infected	% Infection	¹ Immune	² Infected	% Infection
AUA/Rhon	42	0	0.00	-	-	-
Bensheim	45	0	0.00	-	-	-
Cape Verde-0	24	1	4.00	19	5	20.83
Catania	-	-	-	21	3	12.50
Columbia-0	250	5	1.96	20	1	4.76
Da(1)	-	-	-	17	7	29.17
Ellershausen-0	-	-	-	19	5	20.83
Estland	19	2	9.52	14	4	22.22
Greenville-0	11	1	8.33	-	-	-
Isenberg	-	-	-	14	7	33.33
Kaunas-0	-	-	-	20	4	16.67
Kendalville	53	1	1.85	-	-	-
Koln-59	-	-	-	24	0	0.00
Lanark-0	-	-	-	10	8	44.44
<i>Landsberg erecta</i>	348	15	4.13	28	4	12.50
Le Mans-2	-	-	-	19	5	20.83
Limeport	-	-	-	20	4	16.67
Muhlen-0	29	0	0.00	20	4	16.67
Niederzenz-0	36	0	0.00	21	0	0.00
Nossen-0	38	0	0.00	-	-	-
Oystese-0	-	-	-	19	5	20.83
Poppelsdorf-0	-	-	-	20	4	16.67
RLD1	30	1	3.23	-	-	-
S96	37	1	2.63	-	-	-

Table 3 List of CAPS markers polymorphic between Arabidopsis ecotypes Col-0 and Nd-0.

CAPS marker	¹ Restriction enzyme	² Primers
1H1L-1.6	<i>Rsa</i> I, <i>Tsp</i> 509I	F:CTAGAGCTTGAAAGTTGATG R:TTGAGTCCTTCTTGTCTG
20B4L-1.6	<i>Dde</i> I	F:CTAAGATGGGAATGTTGG R:GAACTCATTGTATGGACC
40E1T7	<i>Acc</i> I	F:GGTCCACTTTGATTCAAGAT R:GCAAGCGATAGAACATAACG
AF2	<i>Dde</i> I	F:TCGTTCGTTTTTGTTCCTTTTTCTTA R:CCATTCATTTAGGCCCGACTTTC
B9-1.8	<i>Taq</i> I	F:CATCTGCAACATCTTCCCCAG R:CGTATCCGCATTTCTTCACTGC
CAT	<i>Taq</i> I, <i>Tsp</i> 509I	F:GACCAGTAAGAGATCCAGATACTGCG R:CACAGTCATGCGACTCAAGACTTG
ER	<i>Dde</i> I	F:GAGTTTATTCTGTGCCAAGTCCCTG R:CTAATGTAGTGATCTGCGAGGTAATC
G4026	<i>Taq</i> I, <i>Rsa</i> I	F:GTACGGTTCTTCTTCCCTTA R:GGGGTCAGTTACATTACTAGC
G4711	<i>Dde</i> I	F:CCTGTGAAAAACGACGTGCAGTTTC R:ACCAAATCTTCGTGGGGCTCAGCAG
GPA1.1	<i>Tsp</i> 509I	F:ATTCCTTGGTCTCCATCATC R:GGGATTTGATGAAGGAGAAC
JM411	<i>Dde</i> I	F:GCGAACCCTAAGAACTA R:CTCGACTTTGCCAAGGAT
LFY3	<i>Rsa</i> I	F:GACGGCGTCTAGAAGATTC R:TAACCTATCGGGCTTCTGC
MI342	<i>Tsp</i> 509I	F:GAAGTACAGCGGCTCAAAAAGAAG R:TTGCTGCCATGTAATACCTAAGTG
M555	<i>Acc</i> I	F:CCTTTAATTAGTTATCAAATC R:CTCTTGAATTATTAAGTTGACTAG
M59	<i>Rsa</i> I, <i>Tsp</i> 509I	F:GTGCATGATATTGATGTACGC R:GAATGACATGAACACTTACACC
MBK23A	<i>Taq</i> I	F:GATGATTAGGCGCAAAATTGAG R:ATTACCAGCCTGGCTTCAGG
PAI1.1	<i>Taq</i> I, <i>Rsa</i> I, <i>Tsp</i> 509I	F:GATCCTAAGGTATTGATATGATG R:GGTACAATTGATCTTCACTATAG
T20D161	<i>Taq</i> I, <i>Rsa</i> I, <i>Tsp</i> 509I	F:CGTATTTGCTGATTCATGAGC R:ATGGTTTACACTTGACAGAGC
T6P5-4.8	<i>Rsa</i> I	F:TGAAAGACACCTGGGATAGGC R:CCAACCTTTCGGGTCGGTTCC

¹Restriction endonucleases used for individual CAPS markers are shown.²Primers: F, forward primer; R, reverse primer.

Table 4 Sequence Based Polymorphic (SBP) markers generated for the *PSSI* region.

Name	¹Primer	²Enzyme	³Size (bp)
SBP_22.95	F: GGAGGTTCCGTTACTCTTACTG R: CCACCGGAAGACGACGACTCTTC	<i>RsaI</i>	309
SBP_22.98	F: CGACGTCACACTCTCCGTTA R: CCGATGATGGAGAAGGAAAA	<i>TaqI</i>	230
SBP_23.06	F: AAATTGGGGACACCAACAAA R: GGTCTCCTGGGAGAAAGAT	<i>Tsp509I</i>	180
SBP_23.09	F: TCGAATGATCCTTTCCTTTCA R: GCTTTTGCGAAAATGGGATA	<i>TaqI</i>	235
SBP_23.46	F: GACCAAATGTCTCTGAGATGTTC R: ACCCAAGGCGGTGTTGGCGAAAG	<i>TaqI</i>	520

Figures

Figure 1

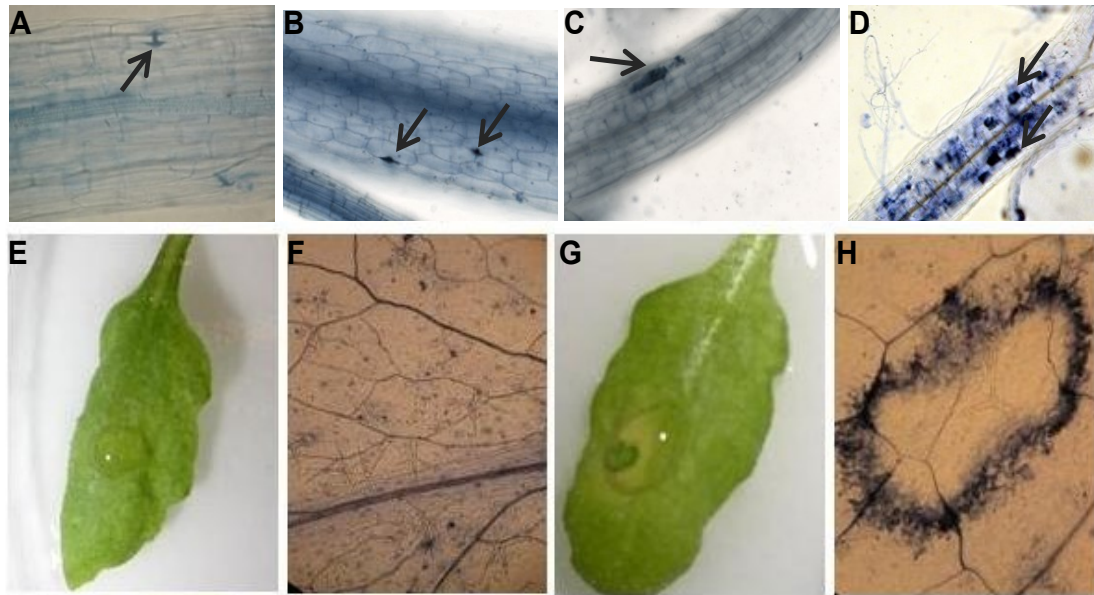
Identification of the *pss1* mutant

Figure 2 A

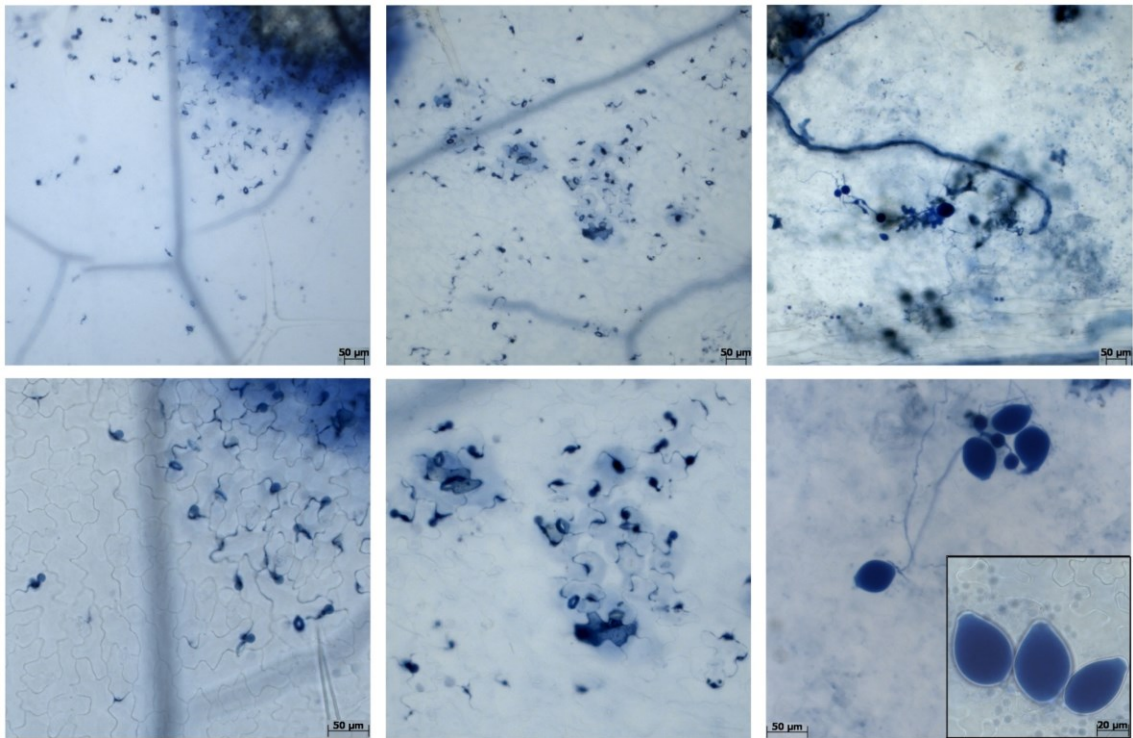
Responses of the *pss1* mutant following *P. sojae* infection

Figure 2 B

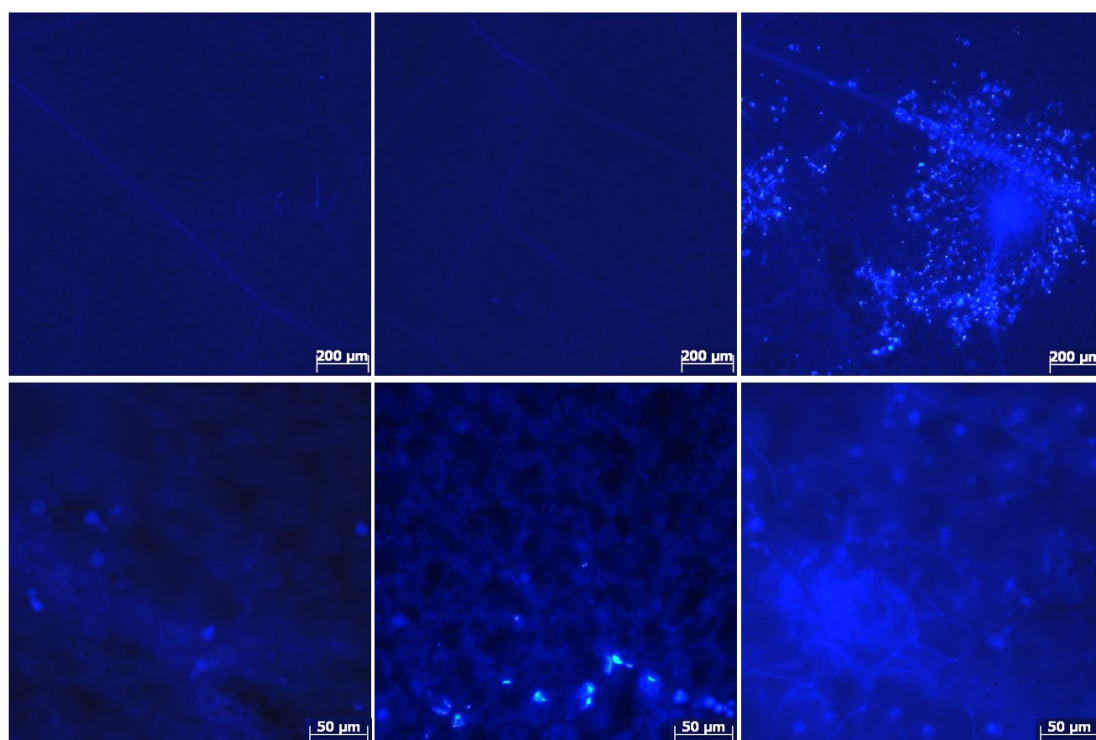
Responses of the *pss1* mutant following *P. sojae* infection

Figure 3

Segregation of *PEN1* alleles among 20 $F_{2:3}$ families homozygous for *pss1*

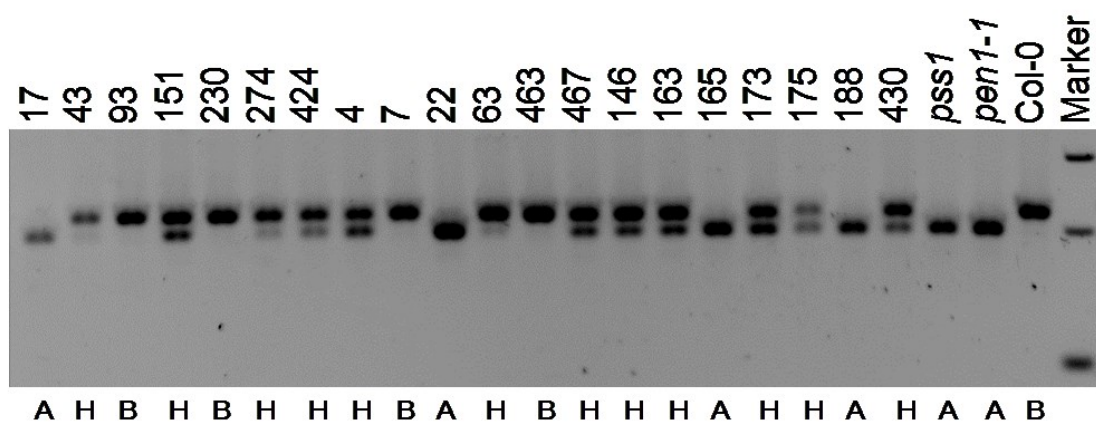


Figure 4

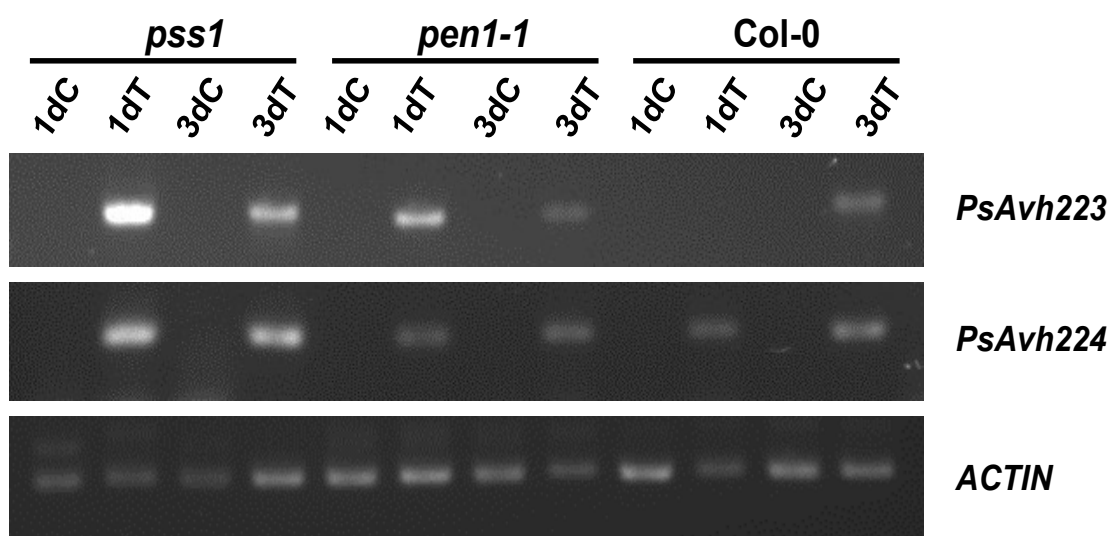
Induction of the effector genes in the Arabidopsis and *P. sojae* interactions

Figure 5

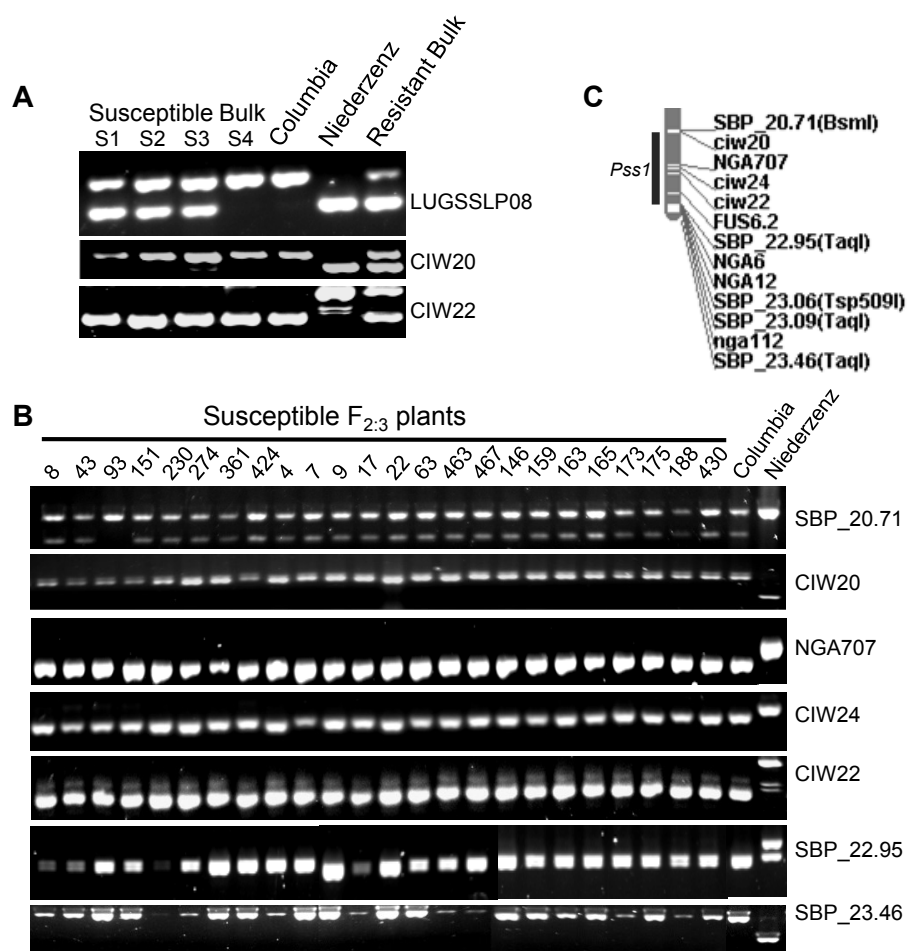
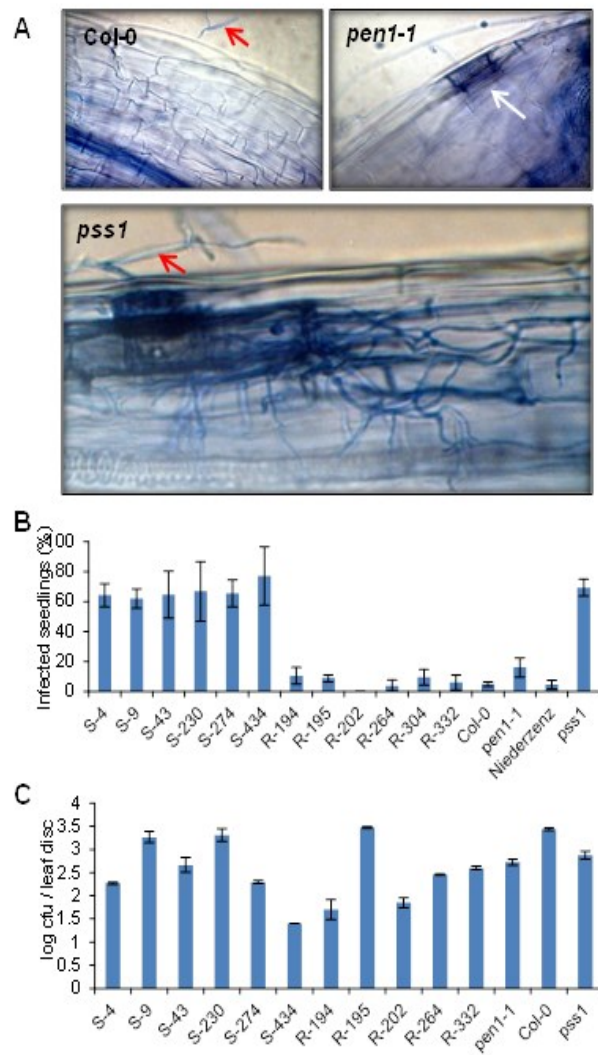
Molecular mapping of the *PSS1* locus

Figure 6

The *pss1* mutant was infected by necrotrophic fungal pathogen, *F. virguliforme*, but not by the bacterial pathogen, *P. syringae* pv. *glycinea*



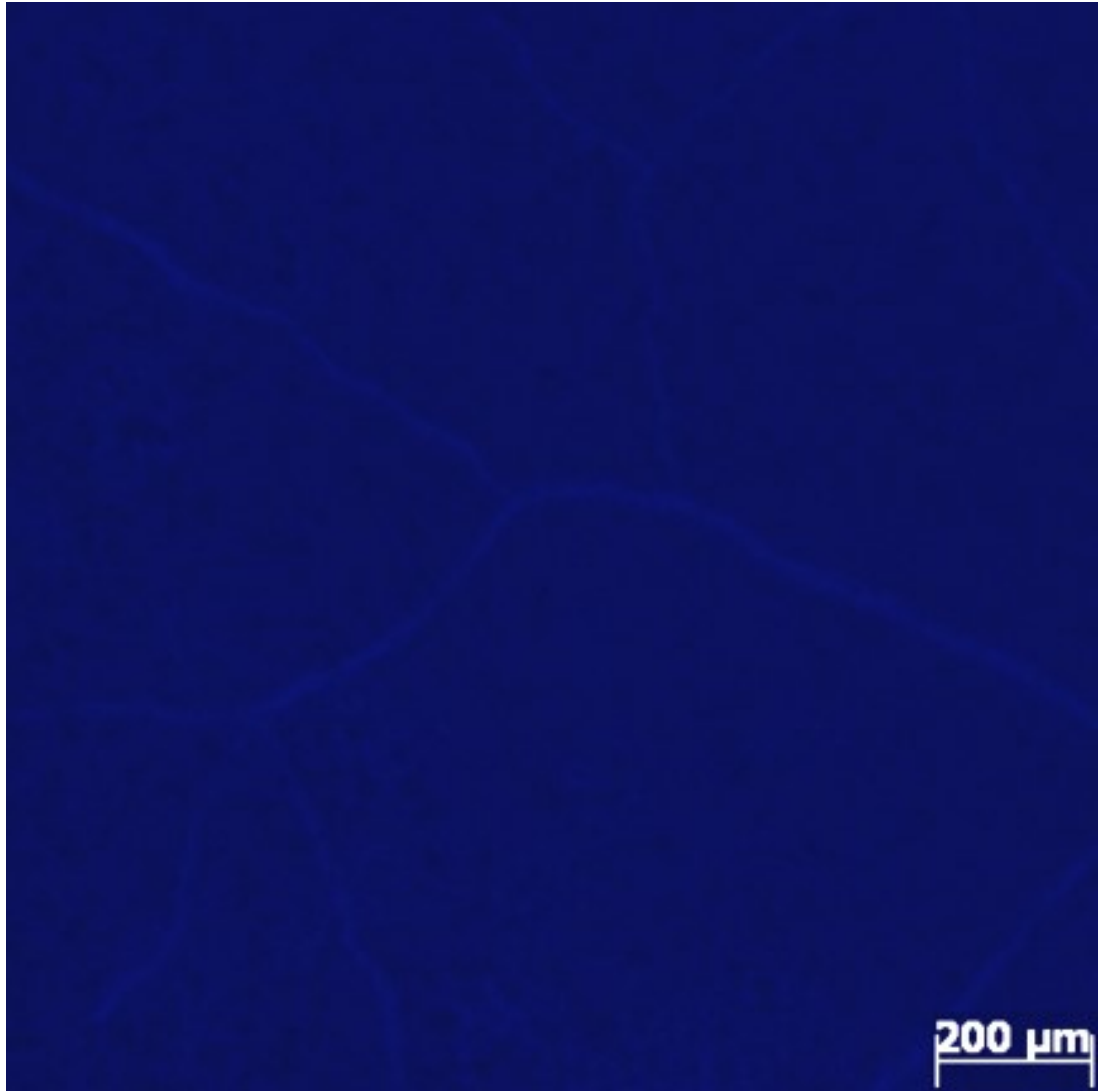
Additional files

Additional File 1

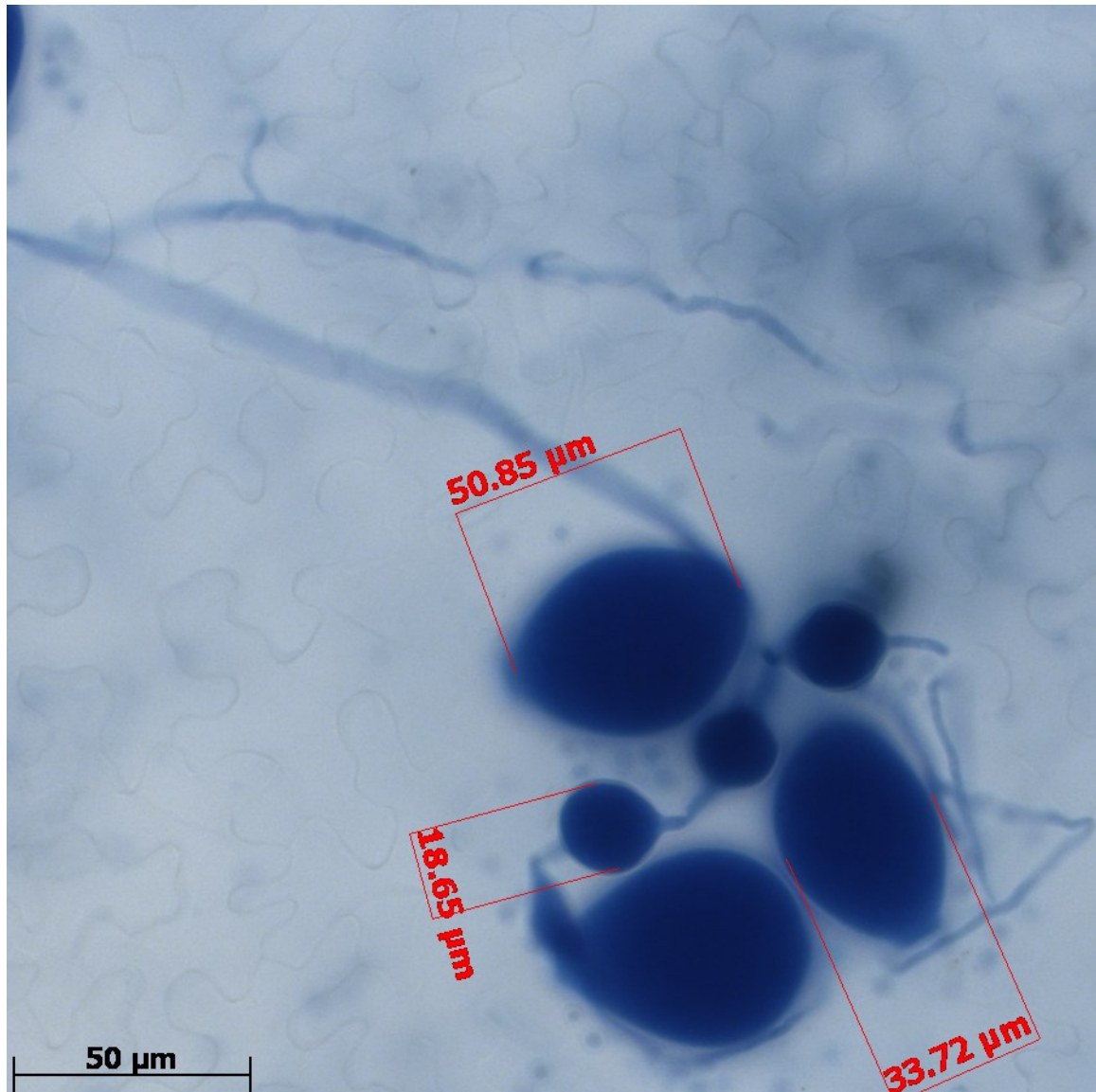
EMS mutants created in *Arabidopsis thaliana pen1-1* mutants showed chlorophyll-lacking mutants among 5% of the M_{2:3} families



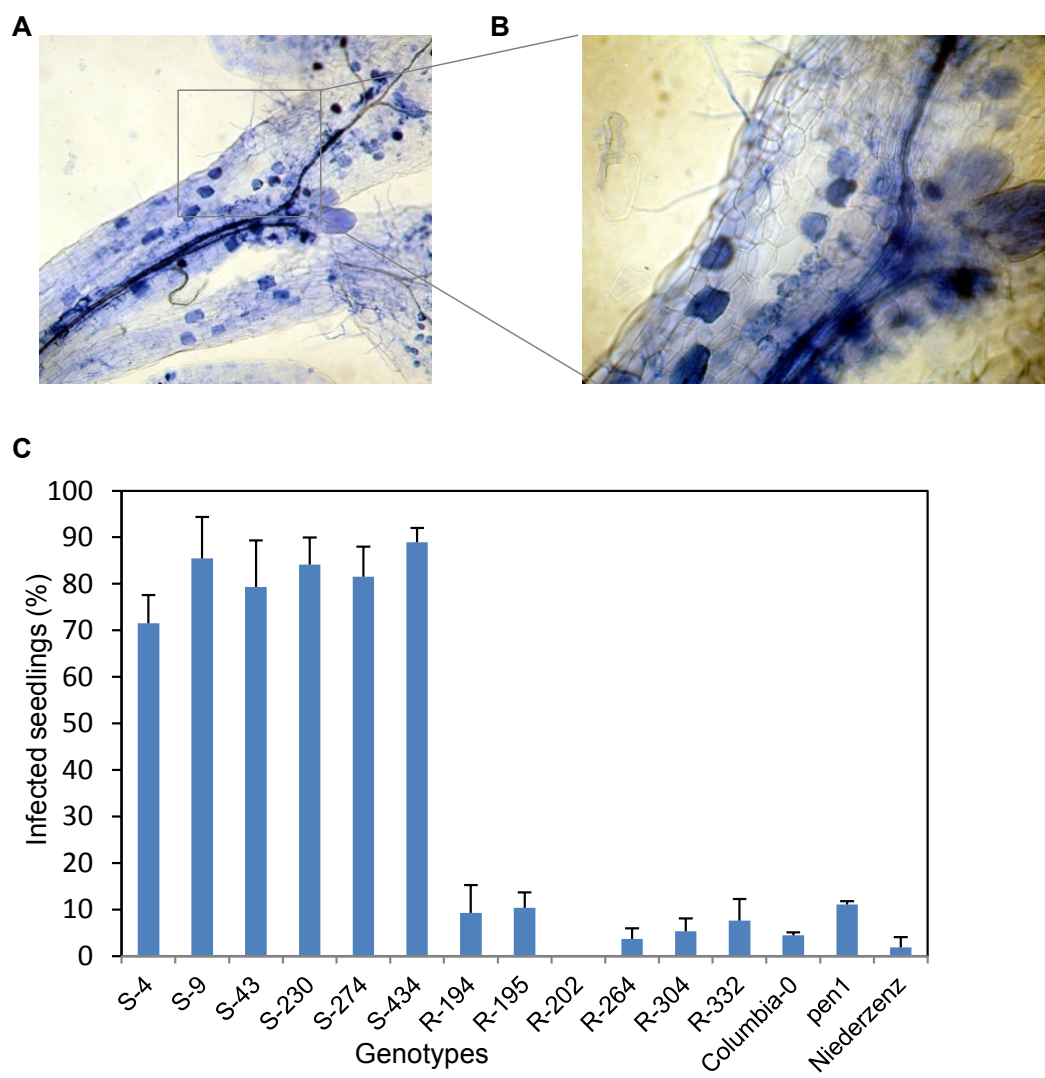
Additional File 2A

Autofluorescence of *pss1* mutant leaf

The *pss1* mutant is a host for soybean oomycete pathogen, *P. sojae*

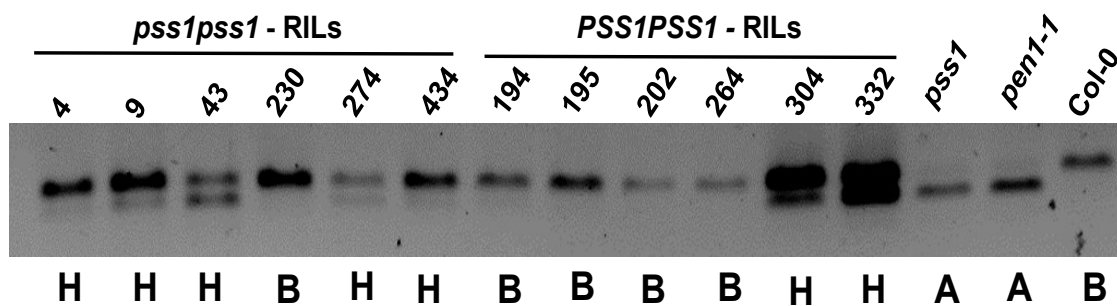


Additional File 3

Identification of F_{2:3} families homozygous for alleles at the *PSS1* locus

Additional File 4

Genotype of six *P. sojae* susceptible (*pss1pss1*) (S-4 through S-434) and five resistant (*PSS1PSS1*) (R-194 through R-332) F_{2:3} families and the *pss1* mutant for the *PEN1* alleles



Additional file 5

Primers used in the RT-PCR experiment

Gene	¹Primer	²Amplicon
<i>PsAvh223</i>	F:GGCCACCCACACACCCCTCCCTCCCGTC R:CGGCGTCCTCGGCCTCGTCGTCTAG	237
<i>PsAvh224</i>	F:GCGCGGCCTCGAGTTCCTTCTTCGTG R:CCTCCCTCCCGTCCGCTACAGTCATG	355
<i>AtActin</i>	F:GGCGATGAAGCTCAATCCAAACG R:GGTCACGACCAGCAAGATCAAGACG	491

Figure Descriptions

Figure 1 Identification of the *pss1* mutant.

A, Columbia-0 and **B**, *nho1* seedlings were not penetrated by *P. sojae*. **C**, *pen1-1* seedling was penetrated by *P. sojae* causing death of a single cell. **D**, The *pss1* mutant showed penetration and colonization by *P. sojae*, thus indicating a loss of NHR against this pathogen. Images shown in A, B, C and D were taken at 100X magnification. Arrows in A and B show failed attempts of penetration by germinating zoospores. Arrows in C and D show the cell death caused penetrating hyphae. **E** and **G**, macroscopic and microscopic responses of *pen1-1* following *P. sojae* infection; **F** and **H**, macroscopic and microscopic responses of *pss1* leaf following *P. sojae* infection. Images shown in F and H were taken at 50X magnification. The photographs show representative results obtained from three independent experiments. Microscopic images of A, B, C, D, F and H were taken following staining of infected tissue samples with trypan blue.

Figure 2 Responses of the *pss1* mutant following *P. sojae* infection.

A, Leaves of 21 day old Col-0, *pen1-1*, *pss1* seedlings were inoculated with *P. sojae* spores and stained with aniline blue dye and visualized under a Zeiss Axioplan II compound microscope with ultraviolet epifluorescence [25]. (i) and (iv), Col-0; (ii) and (v), *pen1-1*; and (iii) and (vi), *pss1* leaves that were sampled 6 hours post inoculation with *P. sojae* spores (1×10^5 spores/ml) to stain with aniline blue for detecting callose deposition. Only *pss1*, but neither *pen1-1* nor Col-0, showed enhanced callose deposition at the penetration sites, as visualized by epifluorescence of the aniline blue (i-iii), 50X magnification; and (iv-vi) 200X magnification. Arrows indicate sites of callose deposition (ca) and secondary hyphae (sh). The experiment was repeated twice with

similar results. **B**, Leaves of 21 day old Col-0, *pen1-1*, *pss1* seedlings were inoculated *P. sojae* spores and stained with trypan blue dye and visualized under a Zeiss Axioplan II compound microscope under bright field illumination [23]. (i) and (iv), Col-0; (ii) and (v), *pen1-1*; and (iii) and (vi), *pss1* leaves that were sampled 7 days post inoculation with *P. sojae* zoospores to stain with trypan blue for detecting cell death and fungal structures. The *pss1* mutant but not *pen1-1* or wild-type Col-0 showed extensive primary and secondary hyphal growth upon infection with *P. sojae*. Arrows indicate various fungal structures; the female reproductive structures, oogonia (oo), sporangia (sp) and secondary hyphae (sh) which were visible on infected *pss1* leaves. (i-iii), 100X magnification; and (iv-vi) 200X magnification. The experiment was repeated twice with similar results.

Figure 3 Segregation of *PEN1* alleles among 20 F_{2:3} families homozygous for *pss1*.

dCAPS marker based on SNP between *PEN1* and *pen1-1* alleles was used to determine the genotypes for alleles of the *PEN1* locus. Genotype A: homozygous for the *pen1-1* allele, B: homozygous for the *PEN1* allele, H: heterozygous.

Figure 4 Induction of the effector genes in the Arabidopsis and *P. sojae* interactions.

Expression levels of two *P. sojae* effector genes, *PsAvh223* and *PsAvh224* highly induced in the soybean-*P. sojae* interactions were measured in a semi-quantitative RT-PCR experiment. Detached leaves of *pss1*, *pen1-1* and Col-0 were inoculated with *P. sojae* or treated with sterile water droplets. The cDNA samples were used to amplify the two effector genes of *P. sojae* and Arabidopsis actin gene. Enhanced expression of both of the effector genes were observed in *pss1* but not in *pen1-1* and Col-0. 1dC, 1 day post water droplet treatment of detached leaves; 3dC, 3 days post water droplet treatment of

detached leaves; 1dT, 1 day post inoculation with *P. sojae* zoospores; 3dT, 3 day post inoculation with *P. sojae* zoospores. Actin was used as an internal control.

Figure 5 Molecular mapping of the *PSSI* locus.

A, Identification of SSLP markers linked to *PSSI*. Similar amplification patterns of SSLP markers CIW20 and CIW22 in susceptible bulks (S1, S2, S3 and S4) and Col-0 suggested that *PSSI* is putatively linked to the two markers. As a control, amplification patterns of a distantly mapped SSLP marker, LUGSSLP08 in the bulk DNA samples are shown. **B**, Co-segregation of *PSSI* with six molecular markers of the south arm of chromosome 3. Twenty-two susceptible $F_{2:3}$ families except one, $F_{2:3}$ family 93, showed same amplification patterns as in Col-0 for these markers. $F_{2:3}$ family 93 showed recombination between *PSSI* and SBP_20.71. **C**, Molecular map of the *PSSI* region. Five SBP markers were developed for the *PSSI* region that was mapped to southern arm of chromosome 3.

Figure 6 The *pss1* mutant was infected by necrotrophic fungal pathogen, *F. virguliforme*, but not by the bacterial pathogen, *P. syringae* pv. *glycinea*.

A, Response of *pss1* to *F. virguliforme* infection. Cell death and spread of mycelia stained with trypan blue dye were observed in infected seedlings of *pss1* but not in those of Col-0 or *pen1-1* following inoculation with *F. virguliforme* conidial spores. Single cell penetration by *F. virguliforme* was observed in *pen1-1* but not Col-0 seedlings. All images were taken 2 days post- inoculation and at 400X magnification. **B**, Responses of six *P. sojae* susceptible (*pss1pss1*) (S-4 through S-434) and six resistant (*PSSI**PSSI*) (R-194 through R-332) $F_{2:3}$ families and the *pss1* mutant to inoculation with *F. virguliforme* conidial spores are presented. Data are the mean of three independent experiments. Error

bars indicate S.E. between experiments. C, Response of *pss1* to *P. syringae* pv. *glycinea*. Disease response in colony forming units (cfu) of six *P. sojae* susceptible (*pss1pss1*) (S-4 through S-434) and five resistant (*PSS1PSS1*) (R-194 through R-332) F_{2:3} families and the *pss1* mutant 2 days following inoculation of intact leaves with *P. syringae* pv. *glycinea* are shown. Data are mean of three replications of a representative experiment. The experiment was repeated two times with similar results. Error bars indicate S.E. between experiments.

Additional file 1: EMS mutants created in *Arabidopsis thaliana pen1-1* mutants showed chlorophyll-lacking mutants among 5% of the M_{2:3} families.

The albino seedlings are shown with arrows.

Additional file 2A: Autofluorescence of *pss1* mutant leaf.

Detached leaf of 21-day old seedlings of the *pss1* mutant were mock inoculated with sterile water and stained with aniline blue and observed under ultraviolet epifluorescence 6 hours post inoculation. The image was taken at 50X magnification. The experiment was repeated three times with similar results.

Additional File 2B: The *pss1* mutant is a host for soybean oomycete pathogen, *P. sojae*.

Detached leaves of *pss1* mutant were inoculated with *P. sojae* zoospores (10⁵ spores/ml.) and stained with trypan blue dye 7 days post inoculation (dpi). Formation of sexual female reproductive structures, oogonia (oo) and asexual reproductive structures,

sporangia (sp) indicate that the pathogen is able to complete its life cycle on the host *pssI* mutant leaves, thus signifying a complete breakdown of Arabidopsis nonhost resistance in this mutant. Numbers indicate the approximate size of the reproductive structures, which is in close agreement with the average size of the reproductive structures of the *Phytophthora* genus [45].

Additional file 3: Identification of F_{2:3} families homozygous for alleles at the *PSSI* locus.

A, Inoculation of a 10 day old *pssI* seedling with *P. sojae* spores followed by staining with trypan blue dye showed extensive hyphal growth and subsequent cell death. Image (100X magnification) was taken at 2 dpi. **B**, The indicated section of A at a higher magnification. **C**, Responses of 10-day old seedlings of six F_{2:3} families, homozygous for the *pssI* allele (S-4 through S-434), and six F_{2:3} families, homozygous for the *PSSI* allele (R-194 through R-332), were inoculated with *P. sojae* zoospores. Data are mean of percent seedlings infected from three independent experiments. Error bars indicate S.E. between experiments.

Additional file 4: Genotype of six *P. sojae* susceptible (*pssIpssI*) (S-4 through S-434) and five resistant (*PSSI**PSSI*) (R-194 through R-332) F_{2:3} families and the *pssI* mutant for the *PEN1* alleles.**

A, homozygous for *pen1-1*, B, homozygous for *PEN1*; H, heterozygous.

Additional file 5: Primers used in the RT-PCR experiment.

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CHAPTER 4. AN ARABIDOPSIS *GLYCINE RICH PROTEIN 1* (*GRP1*) GENE CONFERS RESISTANCE TO *PHYTOPHTHORA SOJAE* IN TRANSGENIC SOYBEAN PLANTS

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RS and BBS performed all the experiments, RS and MKB wrote the manuscript and MKB designed the experiments.

Abstract

Nonhost resistance is the form of resistance in which all members of a plant species are resistant to all isolates of a pathogen which may be virulent on another plant species. Previously, we have identified *Phytophthora sojae* susceptible (*pss1*), a mutant lacking Arabidopsis nonhost resistance against two destructive soybean pathogens, *Phytophthora sojae* and *Fusarium virguliforme*. Here we report that *pss1* encodes an Arabidopsis glycine rich protein (*GRP1*). *GRP1* complemented the *pss1* phenotype and conferred enhanced resistance to both these pathogens in transgenic soybean plants. Our results show the successful inter-species transfer of a novel Arabidopsis nonhost resistance gene, *GRP1* which confers resistance against a hemi-biotrophic oomycete pathogen of soybean (*Glycine max* L. Merr.).

Introduction

Soybean is one of the most economically important crops in the U.S. and a major legume crops worldwide. Soybean is a rotation crop with corn which fixes nitrogen through its association with *Rhizobium* bacterium. Soybean is an important source of

proteins and oil for both human and animal consumption. Soybean is also used for biofuel production. In 2010, the total production of soybean in the United States was valued at over \$38.9 billion, and the US exports of soybean and its products amounted to over \$23 billion [1]. However, it fails to meet full yield potentiality due to damages by various pathogens. For example, the total soybean yield suppression due to pathogens in the United States during the year 2010 was valued at \$5.59 billion [1].

Soybean root and stem rot is a well-known soybean disease that is caused by *Phytophthora sojae*, one of the most destructive pathogens of soybean [1]. The pathogen, formerly known as *Phytophthora megasperma* f. sp. *glycinea*, causes both pre-emergence and post-emergence damping off in seedlings [3]. The pre-emergence damping off symptoms include rotting of germinating seedlings. If infected seedlings emerge from soil, the lower taproot becomes soft and brown and discoloration extends to the hypocotyls [3]. The disease was first identified in Indiana, USA in 1948 and its causal agent identified in 1958 [4].

Previously, we have identified the Arabidopsis *Phytophthora sojae* susceptible (*pss1*) mutant as one of the mutants showing loss of Arabidopsis non-host resistance against the soybean oomycete pathogen, *P. sojae*. This mutant shows enhanced susceptibility against the fungal pathogen, *Fusarium virguliforme*, but not against a bacterial pathogen, *Pseudomonas syringae* pv. *glycinea*, both of which cause diseases in soybean [5].

Here we present data demonstrating that *PSSI* encodes an Arabidopsis glycine rich protein, GRP (named as GRP1, At3g59640) and the transformation of *Arabidopsis*

thaliana GRP1 gene into Williams-82 cultivar of soybean confers enhanced resistance to transgenic soybean leaves against a destructive pathogen, the root and stem rot pathogen, *P. sojae*.

Material and Methods

Plant material and growth conditions

For the leaf inoculation, the seeds were sown on LC1 soil-less mixture (Sun Gro Horticulture, Bellevue, WA) under a 16 h light/8 h dark regime at 21°C with approximately 60% relative humidity. The light intensity was maintained at 120-150 $\mu\text{E}/\text{m}^2/\text{s}$ [5]. Ten days after sowing, the seedlings were transplanted into a new LC1 mixture. The newly transplanted seedlings were covered with humidity domes for two days and thereafter watered every fourth day. A fertilizer mixture of 15:15:15::N:P:K (1% concentration v/v) was applied to the seedlings seven days after transplantation. Seeds of RIL lines of *pss1* mutants, SALK T-DNA insertion lines of *Arabidopsis thaliana* along with the wild type ecotypes, Col-0 and Nd-0, were grown as described in Sumit *et al*, 2012.

Fine mapping and cloning of *PSSI*

Leaf tissue of seven homozygous susceptible *pss1pss1* F_{2:3} families were bulked to prepare genomic DNA using the CTAB method. This *pss1* genomic DNA was sequenced using Illumina/Solexa sequencing. After the paired end sequencing of the genome it was assembled and aligned to the reference Col-o sequence by using the SHORE program (Ossowski *et al*, 2008). The CS850460, SALK_148857C line having T-DNA insertion in the promoter and SALK_090245C in the exons of AT3G59640;

glycine-rich protein showed susceptibility to infection by the *P. sojae* as in the *pssI* mutant. After the glycine rich protein (GRP1) was confirmed to be the *PSSI* gene, the cDNA sequence (741bp; *At3g59640*) was amplified from the wild type Col-0 cDNA sample. The *GRP1* gene was cloned in the pISU-Agron5 under 2X35S promoter at *Bam*HI site and was transformed in DH10B. After the correct orientation checking by restriction digestion and sequence confirmation, the construct containing the *GRP1* gene was transformed in EHA101 and was used for complementation of the *pssI* mutants and the SALK lines. In addition, the construct having the *GRP1* transgene was also sent to the Plant transformation facility at Iowa State University to transform the Williams 82 soybean plants. Basta resistant plants were selected to characterize them further.

Transgenic soybean evaluation by leaf inoculation with *P. sojae*

The transformed plants with basta resistance were grown to harvest the T₁ seeds for testing the phenotype of the complementation analysis. 13 day old complemented plants were inoculated by using the CC5C isolate of *P. sojae* with the respective controls and the disease symptoms were monitored timely. Soybean transformants having the basta resistance were grown in the growth chamber with light intensity (600 $\mu\text{mol}/\text{cm}^2/\text{sec}$) for 13 days and the unifoliate leaves were selected for the infection experiment. Three different isolates of *P. sojae* i.e. NW5A, R-1012 and CC5C (10^5 spores/mL) were used for leaf inoculation of transgenic soybean plants. Observations were recorded by measuring the lesion length at 1, 2 and 3 dpi for *P. sojae*. Detached leaves of wild type cultivar Williams-82 were used as control.

Results

Arabidopsis nonhost resistance gene *PSSI* encodes a glycine rich protein (GRP1)

To facilitate cloning of *PSSI* a DNA sample of seven *pss1pss1* homozygous families was sequenced by applying the Solexa sequencing technology at the Iowa State University DNA Facility. By comparing sequences of the *PSSI* region of the bulked susceptible F_{2:3} families homozygous for the *pss1* allele with that of the ecotype Col-0 with the aid of the SHORE program [6], we identified SNPs of the region that carries the *PSSI* locus. This 2.75 Mb region is located in the lower arm of Chromosome 3 starting from 20.71Mb to 23.46 Mb [5]. Among the SNPs of 30 genes in this region, 10 were found to be non-synonymous and were further studied. To identify SNPs originating from EMS treatment of *pen1-1* seeds, we eliminated the mutations found in the *pen1-1* background. We observed that among the nine mutations specific to the *PSSI* region, three were originated from the *pen1-1* mutant background and therefore are not candidate *PSSI* genes were not considered for further investigation. The remaining six genes carried novel point mutations due to EMS treatment of the *pen1-1* seeds and were considered as putative *PSSI* genes (Table 1). We investigated if any of the candidate *PSSI* genes have homologous in the soybean genome by conducting BLAST search at the NCBI website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The results are presented in Table 2. We observed that none of the six candidate *PSSI* genes are conserved in soybean. Thus, it is possible that orthologous *PSSI* sequence is missing in soybean and transfer of *PSSI* to soybean may result in enhanced *Phytophthora* resistance in soybean.

The SALK T-DNA insertion lines for these six genes were analyzed for their response to *P. sojae* infection. We investigated if those SALK lines were homozygous for

the T-DNA insertion (Figure 2). In order to identify the mutant genotypes carrying T-DNA insertion mutations in homozygous condition, PCR was performed using the T-DNA left border-specific primer and the gene specific forward and reverse primers. Twenty-eight T-DNA mutants with insertion in five candidate *PSSI* genes were collected from ABRC (<http://abrc.osu.edu/>) and analyzed for T-DNA insertion and the disease phenotypes following *P. sojae* infection. CS850460, SALK_148857C and SALK_090245C lines carrying T-DNA insertion mutations in the *At3g59640* gene which encodes a glycine rich protein were consistently infected by *P. sojae*. We termed the *At3g59640* gene tentatively as *GRP1*. The homozygous T-DNA mutants in the *GRP1* gene identified from CS850460, SALK_148857C and SALK_090245C lines were termed *grp1-1*, *grp1-2* and *grp1-3*, respectively (Figure 1). We characterized the CS850460 SALK T-DNA insertion line response to *P. sojae* inoculation and observed a phenotypic response similar to the *pss1* mutant and the homozygous susceptible *pss1*-RILs (Figure 2). Thus, Arabidopsis glycine rich protein (*At3g59640*) was likely the *PSSI* gene. The *grp1-1*, *grp1-2* and *grp1-3* mutant lines were verified for T-DNA insertion and were found to carry the T-DNA insertion following PCR with T-DNA specific primers (Figure 3). Following this, we confirmed that the *grp1-1*, *grp1-2* and *grp1-3* mutant lines did not show expression of the *GRP1* gene (Figure 4) and this lack of expression correlated strongly with the disease phenotype of the mutants upon inoculation with *P. sojae*. The lines carrying T-DNA insertion mutations for remaining four candidate *PSSI* genes segregated for the T-DNA insertions, but were resistant to *P. sojae* (data not presented) and therefore these four genes cannot be candidate *PSSI* genes. GK-476F03 line was obtained from NASC (<http://arabidopsis.info/>). It contains a T-DNA insertion in the sixth

candidate *PSSI* gene, *AT3G59650*. The mutant was resistant to *P. sojae*; therefore, *At3g59650* is not a candidate *PSSI* gene. Thus, the T-DNA insertion analyses indicate that *PSSI* encodes a glycine rich protein (GRP1, *At3g59640*), which we named as GRP1.

Transformation of Arabidopsis glycine rich protein 1 gene (*GRPI*) confers enhanced resistance against *P. sojae* in transgenic soybean plants.

To confirm that Arabidopsis glycine rich protein 1 (GRP1, *At3g59640*) is encoded by the *PSSI* gene, the cDNA sequence of the gene (741bp; *At3g59640*) was amplified from the wild type Col-0 cDNA sample. The *GRPI* gene was cloned in the pISU-Agron5 vector under 2X35S, constitutive promoter at the *Bam*HI site and was transformed into DH10B cells. The construct containing the cDNA sequence of the *GRPI* gene was transformed into the *Agrobacterium tumefaciens* EHA101 strain for transformation of the *pss1* mutants and the SALK lines. In addition, the construct having the cDNA sequence of Arabidopsis *GRPI* gene was also transformed into the wildtype cultivar Williams 82 soybean plants at the plant transformation facility, Iowa State University. Herbicide glufosinate-ammonium (BastaTM) was used to select the transgenic plants and the transformed plants carrying BastaTM resistance were grown to harvest the T₁ seeds. The 21-day old progenies of the transformed plants were infected with *P. sojae* CC5C, R1012 and NW5A isolates and the disease symptoms were monitored. Our results indicate that the transfer of novel Arabidopsis nonhost resistance gene, *GRPI* conferred enhanced resistance against the oomycete pathogen, *P. sojae* among transgenic soybean plants (Figure 5).

Progenies of transgenic soybean plants carrying Arabidopsis *GRP1* gene show heritable resistance to *P. sojae*.

Seeds of *P. sojae* resistant plants carrying the Arabidopsis *GRP1* transgene were harvested. Similarly, plants from lines that did not carry the Arabidopsis transgene and showed a susceptible phenotype upon leaf inoculation with *P. sojae* were also harvested. Progeny seeds of these *P. sojae* resistant and susceptible lines were then leaf inoculated in the same manner as described above. This experiment was conducted to verify if the transgene was inherited in the progeny population and if the presence of this transgene conferred *P. sojae* resistance in the next filial generation. We investigated progenies of four resistant and four susceptible plants. The parent plants from all three original transgenic events, namely, ST154-13, ST154-21 and ST154-28 were also included in this experiment as control. Our results from this leaf inoculation experiment suggest that the Arabidopsis nonhost resistant gene, *GRP1* is stably transformed into the soybean transgenics and confers enhanced resistance against *P. sojae* in progeny generations (Figure 6).

***In silico* analyses of Arabidopsis glycine rich protein (*GRP1*) gene**

Arabidopsis *pss1* mutant protein carries a glycine to aspartate residue mutation at position 119.

The non-synonymous mutation caused by chemical mutagen EMS caused mutation of a G→ A nucleotide in exon 2 of the Arabidopsis *pss1* mutant. To verify if this mutation was a non-synonymous mutation, we aligned the amino acid sequences of *pss1* and wild type GRP1 protein. A single mutation of a glycine at position 119 to an aspartic acid residue caused by this EMS generated mutation.

The GRP1 glycine residue at position 119 is conserved among diverse plant species.

To verify whether the mutated glycine residue at position 119 in the Arabidopsis GRP1 protein is conserved among the homologous or orthologous GRP1 proteins of other species, a CLUSTAL 2.1 alignment analysis was conducted. This alignment showed that the glycine residue (position 119 in the GRP1 protein) is conserved across all proteins included in this study (Table 4). Conservation of the glycine residue at position 119 and substitution of this residue in the *pss1* suggest that this glycine residue plays an important role in providing nonhost resistance of Arabidopsis against the soybean pathogens. However, a PROSITE search to investigate any conserved domains in the GRP1 protein failed to identify any conserved domains in this protein (www.prosite.expasy.org). Also, *GRP1* was not found to share any sequence similarity to other characterized Arabidopsis glycine rich proteins (GRPs), some of which have been known to play important role in providing resistance against pathogens [7] (Fu et al, 2007). The GRP1 protein is a basic protein with theoretical pI as 10.20 and the molecular weight as 27.14 kDa. The *pss1* mutant protein has a pI value of 10.12 and the molecular weight of 27.19 kDa due to mutation of a hydrophobic glycine residue to a negatively charged aspartate residue.

Discussion

Nonhost resistance is the most common form of plant resistance, yet the molecular mechanisms of this broad-spectrum resistance remain unknown. Previously we have identified *pss1* as one of the mutant from a genetic screen for Arabidopsis mutants showing loss of nonhost resistance against the soybean root rot oomycete pathogen, *P. sojae* [5]. This mutant showed loss of Arabidopsis nonhost resistance also against the

soybean sudden death syndrome pathogen, *F. virguliforme*. Here we present evidences to suggest that *PSSI* encodes an Arabidopsis glycine rich protein (GRP), which we named GRP1.

We identified six candidate *PSSI* genes following sequencing of the seven *pss1pss1* homozygous susceptible F_{2:3} families and analyzing the SNP mutations caused by EMS treatment of Arabidopsis *pen1-1* seeds (Table 1). We then investigated the SALK T-DNA insertion mutants for all the candidate *PSSI* genes for their responses following *P. sojae* inoculation. Two T-DNA mutants carrying non-functional Arabidopsis glycine rich protein (*At3g59640*, named as GRP1) were found to show a susceptible phenotype similar to the *pss1* mutant (Figure 2). Transformation of the *pss1* mutant with the wild type copy of the cDNA sequence of *Arabidopsis thaliana* *GRP1* gene complemented the susceptible phenotype thus indicating that we have identified a novel Arabidopsis nonhost resistance gene (Figure 2). We investigated for any known homologues of the *GRP1* gene in the host plant soybean and found that soybean does not contain any known homologue of this important nonhost resistance gene (Table 3).

Following the successful complementation of the mutant phenotype with the cDNA of the *GRP1* gene, I verified whether this *Phytophthora* resistance provided by *GRP1* gene could also be transferred to the soybean plant. Soybean cultivar, Williams-82 was transformed with the cDNA of the Arabidopsis *GRP1* gene. The progenies of these plants show resistance to multiple isolates of the soybean oomycete pathogen, *P. sojae* (Figure 5 and 6). These results indicate that the Arabidopsis glycine rich protein (*At3g59640*) gene, *GRP1*, provides nonhost resistance against two soybean pathogens.

Our results indicate that transgenic soybean plants containing *Arabidopsis thaliana* GRP1 showed enhanced broad-spectrum resistance to *P. sojae* isolates.

Glycine rich proteins (GRP) are an ancient class of proteins that are well known for their role in providing resistance against fungal or viral pathogens [6-7]. Recently, an *Arabidopsis* glycine rich protein (AtGRP7) has been shown to be involved in both host as well as nonhost resistance in *Arabidopsis* and tobacco, respectively [8]. Recently, Fu et al. [8] identified a glycine rich protein (GRP7) as one of the targets of a bacterial pathogen effector protein of *Pseudomonas syringae* pv. *tomato* (DC3000). The authors identified two arginine residues at position 47 and 49 separated by a serine required for its resistance function[8]. The GRP1 protein also contains two arginine residues separated by a glycine residue at position 124 and 126. In another recent study, homologous effector proteins with a conserved RXLR motif from *H. arabidopsidis* and *P. sojae* were found to suppress immunity in *Nicotiana benthamiana* plants [9]. This study indicated that both pathogens target the same resistance mechanism in tobacco plants.

Glycine rich proteins (GRP) are also well known for their role in providing resistance against fungal or viral pathogens. Most of these proteins carry a signal peptide and other specific structures such as a cold shock response domain or the RNA-binding regions. The antimicrobial role of these proteins has been suggested by up –regulation of their expression upon infection with fungal pathogens. However, not much is known about the mechanism of action of these proteins in providing resistance against filamentous fungal pathogens. Recently, Zottich *et al.*[10] have indicated that an small antifungal peptide with sequence homology to glycine rich proteins plays a role in providing resistance against fungal pathogens in *Coffea canephora* [10]. The authors

showed that a sequence with homology to several glycine rich protein sequences from various plants such as *Medicago sativa* (65% identity) has antifungal activity and also indicated the possible mechanism of action of this peptide sequence. This peptide sequence permeabilizes the fungal plasma membrane and also alters the haustorial morphology of the invading fungus, *Candida lindemuthainum* and *Fusarium virguliforme* [10]. However, a BLAST search of Arabidopsis GRP1 protein did not show any homology with any other glycine rich proteins that have been shown to play a role in disease resistance.

Thus, our results from this study have identified a novel Arabidopsis gene, which plays an important role in conferring Arabidopsis nonhost resistance against the soybean pathogens, *P. sojae* and *F. virguliforme*. The inter-specific transfer of this gene to the host plant soybean conferred enhanced broad-spectrum resistance against the oomycete pathogen, *P. sojae*. Further characterization of this important and novel nonhost resistance gene would help identify the mode of action of a prevalent form of plant resistance, the nonhost resistance.

Tables

Table 1 List of candidate *PSS1* genes carrying novel non-synonymous mutations.

SNPs	LOCUS	GENE NAME	MUTATION IN	BASE CHANGE	A.A. CHANGE	Same mutation in <i>pen1-1</i>
20795011	AT3G55860	udp-GLUCOSE PYROPHOSPHORYLASE	EXON	A-T	ASP/GLY	YES
20795012	AT3G55860	udp-GLUCOSE PYROPHOSPHORYLASE	EXON	T-C	ASP/GLY	YES
22029832	AT3G59640	GLYCINE-RICH PROTEIN	EXON	G-A	GLY/ASP	NO
22033274	AT3G59650	MITOCHONDRIAL RIBOSOMAL PROTE	EXON	G-A	GLY/ASP	NO
22290347	AT3G60310	UNKNOWN PROTEIN	EXON	G-A	ALA/THR	NO
22477739	AT3G60840	MICROTUBULE ASSOCIATED PROTEIN	EXON	G-A	PRO/LEU	NO
22504152	AT3G60920	CONTAINS INTER-PRO DOMAIN	EXON	G-A	ALA/ASP	NO
22786292	AT3G61580	FATTY ACID/SPHINGOLIPID DESATUR	EXON	G-A	ASP/ASN	NO
20817955	At3g56100	MERISTEMATIC RECEPTOR-LIKE KIN	EXON	Insert-T	STOP	YES

Table 2 Soybean homologues of the *PSSI* candidate genes.

Gene ID	Arabidopsis Protein ID	Amino Acid	Soybean Homologue	Identity (%)	E-value
AT3G59640	Glycine-rich protein	246	Unknown Protein	38 (89/235)	1.00E-34
AT3G59650	Mitochondrial ribosomal protein	119	Unknown Protein	27 (22/83)	6.00E-04
AT3G60310	Unknown protein	687	Unknown Protein	27 (29/106)	0.049
AT3G60840	Microtubule assoc. protein 65-4	648	Unknown Protein	24 (19/78)	0.023
AT3G60920	Contains inter-pro domain	1941	Unknown Protein	32 (18/56)	0.01
AT3G61580	Fatty acid/sphingolipid desaturase	449	Unknown Protein	38 (28/73)	6.00E-15

Table 3 Phenotype of three SALK T-DNA insertion mutants in Arabidopsis glycine rich protein (GRP1), At3g59640.

Mutant allele	T-DNA insertion line	Location of insertion	Homozygosity of insertion	Transcripts	Phenotype
<i>grp1-1</i>	CS850460	Promoter	Yes	Absent	Susceptible
<i>grp1-2</i>	SALK_148857C	Promoter	Yes	Reduced	Segregated
<i>grp1-3</i>	SALK_090245C	Exon-1	Yes	Absent	Susceptible

Table 4 A conserved glycine residue is mutated in Arabidopsis mutant, *pss1*.

Sequences of proteins found to have sequence similarity to Arabidopsis GRP1 were aligned using CLUSTAL 2.1 software program. Alyrata: *Arabidopsis lyrata* GRP protein, Capsella 458: *Capsella rubella* hypothetical protein, Atunknown: *A. thaliana* uncharacterized protein, AT2G43630, Alyrata483474: *A. lyrata* hypothetic protein,

Capsella502: *Capsella rubella* hypothetical protein, Prunus: *Prunus persica* hypothetical protein.

CLUSTAL 2.1 multiple sequence alignment

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GRP1      MSSTQANLCRPSLFCARTTQTRHVSSAPFMSSSLRFDYRPLPKLAIRASASSS-MSSQFSP 59
Alyrata   MSSTQANLCRPSLFCARTTQTRHVSASFKSSSLRFDYRPLPKLTIRASASSSSMSTLFSP 60
Capsella458 MSSTQANLFRPSLFCARTTQTRHVSAGAPFKTSLRFDPRPFKLTIRASASSS-VSTRFLP 59
Atunknown  MSFTQANCFRPSYYPARITRPNCISSVPIRSSVRFDHFPRTSFTLRATAAVS---TQFSP 57
Alyrata483474 MSFTQANCFRPSYYPARITRPNCVSSVSIRSSVRFDYCPRTSFTLRATTAVS---TQFSP 57
Capsella502 MSLTQANCFRPSYSPARITRPNCISSVPIRSSAGVDHCPRTTFTLRATASVS---TQFSP 57
Prunus     -----

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GRP1      LQNHRCR----NQRQGPVVCLLGGKDKSNGSNEISSSTWEAIEKAMGKKSVEDMLREQIQK 115
Alyrata   LQNHRCR----NQRQGPVLCCLGGKDKSNGSNEISSPWKAIEKAMGKKSVEDMLREQIQK 116
Capsella458 LQNHGCR----NQKQGPVACLGGKDKSNGSSEISSPWKAMKKAMGRKSVEDMLHEQIQK 115
Atunknown  LLDHRRRLPTGKSKQSSAVCLFGGKDKPDGSDEIS-PWKAIEKAMGKKSVEDMLREQIQK 116
Alyrata483474 LLNHRRLPTGKSKQSPAVCLFGGKDKPDGSDEIS-PWKAIEKAMGKKSVEDMLREQIQK 116
Capsella502 LLNHRRLPTGKSKTSPAVCLFGGKDKPDGSDEIS-PWKAIEKAMGKKSVEDMLREQIQK 116
Prunus     -----MGNLKKD-----QSIEDVLRQQIER 20
              :*. .*                      :*:*:*:*:*:

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GRP1      K-----DTGG-IPPRGRGGGG--GRNGGNNGSGGSSGEDGGLASFGDETLQVVLATLG 166
Alyrata   K-----DTGG-IPPQG-GGGGG--GRNGGNNGSGGSSGEDGGFASFGDETLQVVLATLG 166
Capsella458 N-----DTGG-IPPQGGGGGGGKGDGRNGGNNGSEGSSGDDKGLA---DETLQVVLATLG 166
Atunknown  KDFYDTDSGGNIPPRGGSGGG--GGNGEER-PEGSGGEDGGLAGIADETLQVVLATLG 172
Alyrata483474 KDFYDTDSGGNIPPRGG-SGG--GGNGEER-PEGSGGEDGGLAGIADETLQVVLATLG 171
Capsella502 KDFYDTDSGGNIPPRGGSGGG--GGNGEER-PEGSGGEDGGLAGIADETLQVVLTGL 172
Prunus     NEFYE-ERCG-----GGGGGGSGSGRSGGDTGGSGSEDEGLAGIMDETLQVILATVG 74
              :      : **      * .*** * .. . *...: * *: * *****:*.*:

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GRP1      FIFLYFYIINGEELFRLARDYIRYLIGRPKSVRLTRVMEGWSRFFEKMSRKKVYNEYWLK 226
Alyrata   FIFLYIYIINGEELFRLARDYIRYLIGRPKSVRLTRVMEGWSRFFERMSRKRKYNEYWLK 226
Capsella458 FIFLYIFIIDGEELFRLARDYIRYLIGRPKSVRLTRVMESWSRFFESLLRKRKYNEYWIK 226
Atunknown  FIFLYTYIITGEELVKLARDYIRFLMGRP KTVRLTRAMD SWNGFLEKMSRQRVYDEYWLE 232
Alyrata483474 FIFLYTYIITGEELVKLARDYIRFLMGRP KTVRLTRAMD GWNGFLEKMSRQRVYDEYWLE 231
Capsella502 FIFLYTYIITGEELVKLARDYIRFLTGKSKTVRLTRAMD GWNEFFEKMSRQRVYDEYWLE 232
Prunus     FLFLYFYIISGEWTRLAKDYIKFLLSGSKSIRLQSRMYKWGRFYKNLTEKKYDKFWLE 134
              *:*** :** *** :*:***:*. .*:** * * * . * : : .: :*:*:

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GRP1      IKRSST-----SLPGLVTRANTNAS----- 246
Alyrata   IKRSST-----RLPGMITRANTDAS----- 246
Capsella458 IKRSSR-----SLPGMIAPTNTDAS----- 246
Atunknown  KAIINTPTWYDSPEKYRRVIKAYVDSNSDEAYVESNSDEVS 274
Alyrata483474 KAIINTPTWYDSPEKYRRVIKAYVDSNSDEAYVDSNSDEVS 273
Capsella502 KAIINTPTWYDSPEKYRRILSAYVDSNSNEDYVDANSDD--- 271
Prunus     KAIITPTWWDSPKYRHIVRSNLESNSDE----- 164
              .              :      :*:

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Figures

Figure 1

Introduction of the wild-type allele of the glycine rich protein 1 (GRP1) restores resistant phenotype and nonhost resistance of the T-DNA mutant CS850460 for *GRP1*

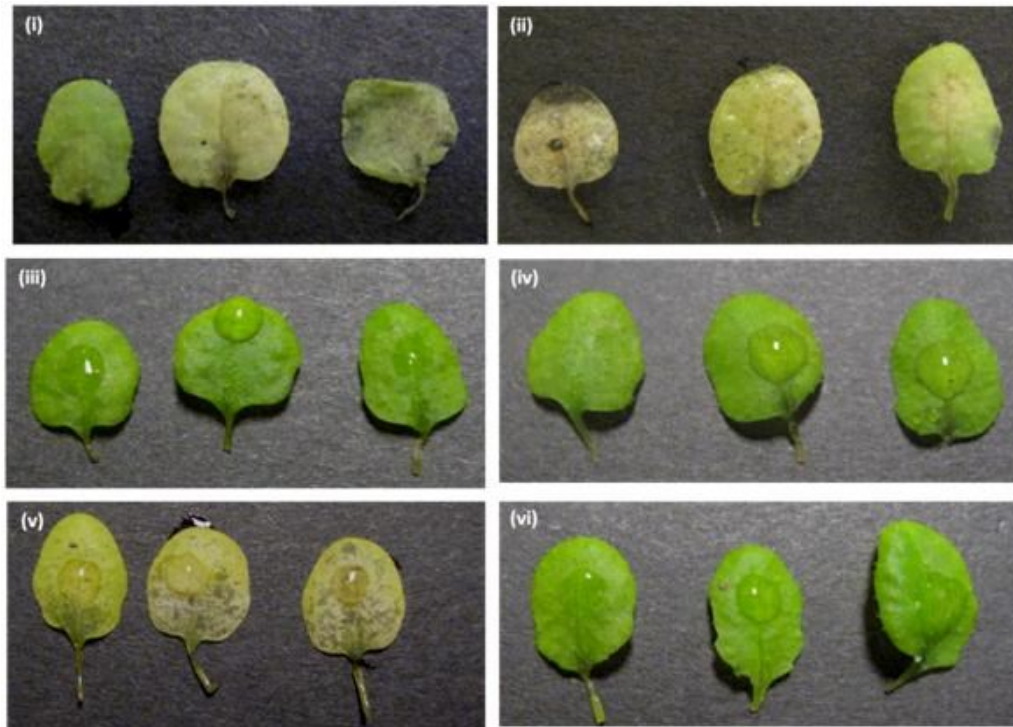


Figure 2

Arabidopsis GRP1 restores the wild type resistant phenotype of the *pss1* mutant following *P. sojae* inoculation

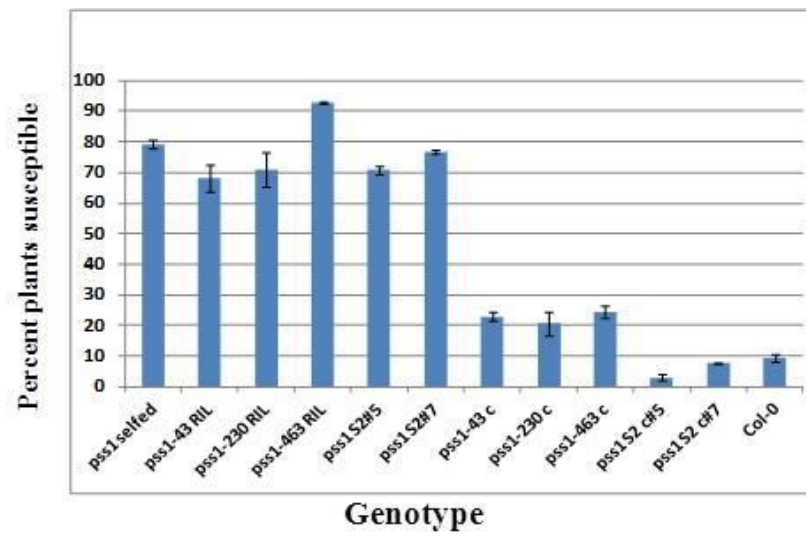


Figure 3

Characterization of SALK lines carrying T-DNA insertions in the candidate *Pss1* gene (*At3g59640*) encoding a glycine-rich protein

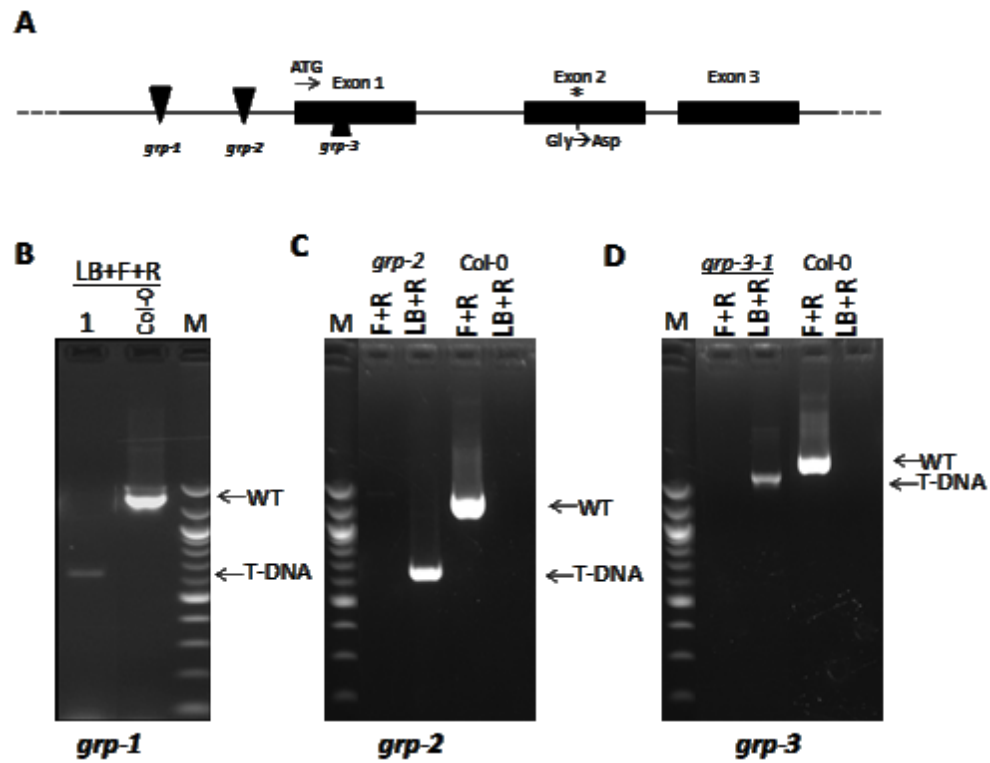


Figure 4

Expression analysis of the *At3g59640* gene in the homozygous SALK T-DNA insertion lines

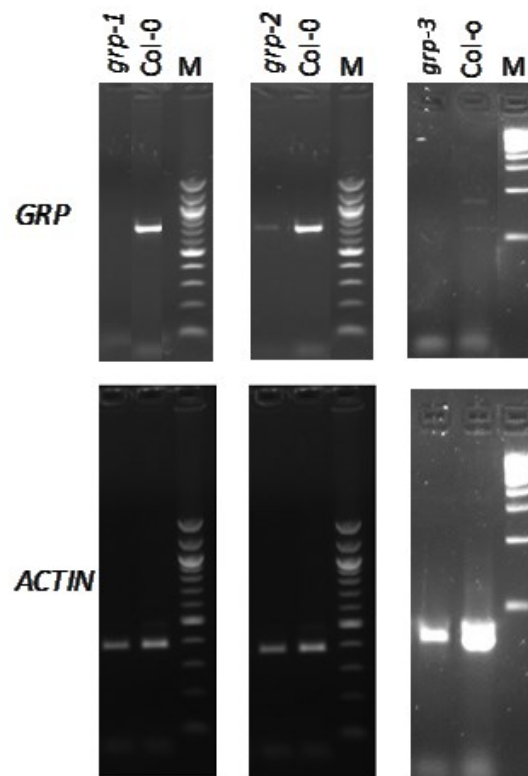


Figure 5

Enhanced broad-spectrum resistance of transgenic soybean plants carrying *Arabidopsis PSS1* to the soybean root rot pathogen, *Phytophthora sojae*

A



B

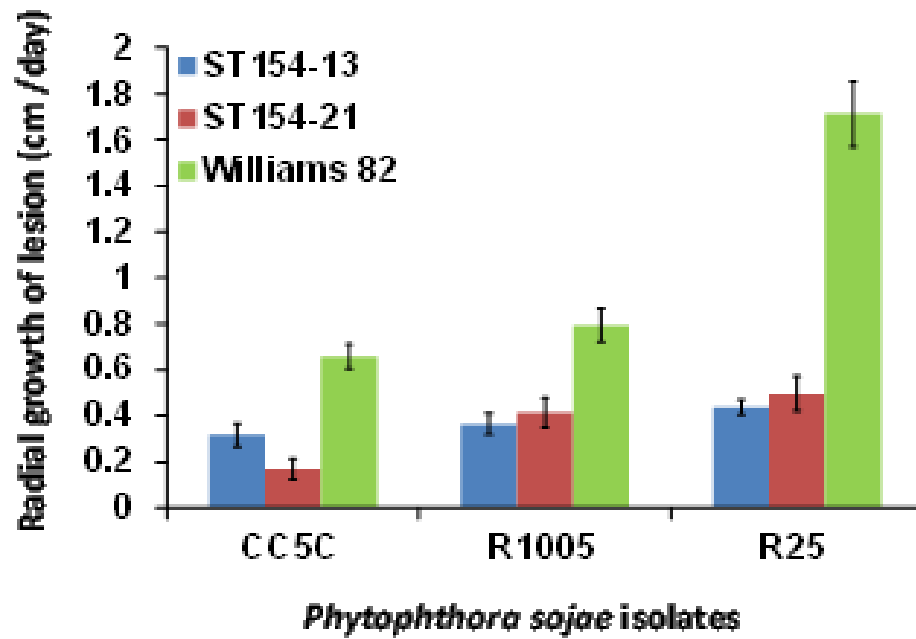


Figure 6

Progeny of transgenic soybean plants carrying Arabidopsis GRP1 gene also show enhanced resistance to soybean oomycete pathogen, *Phytophthora sojae*

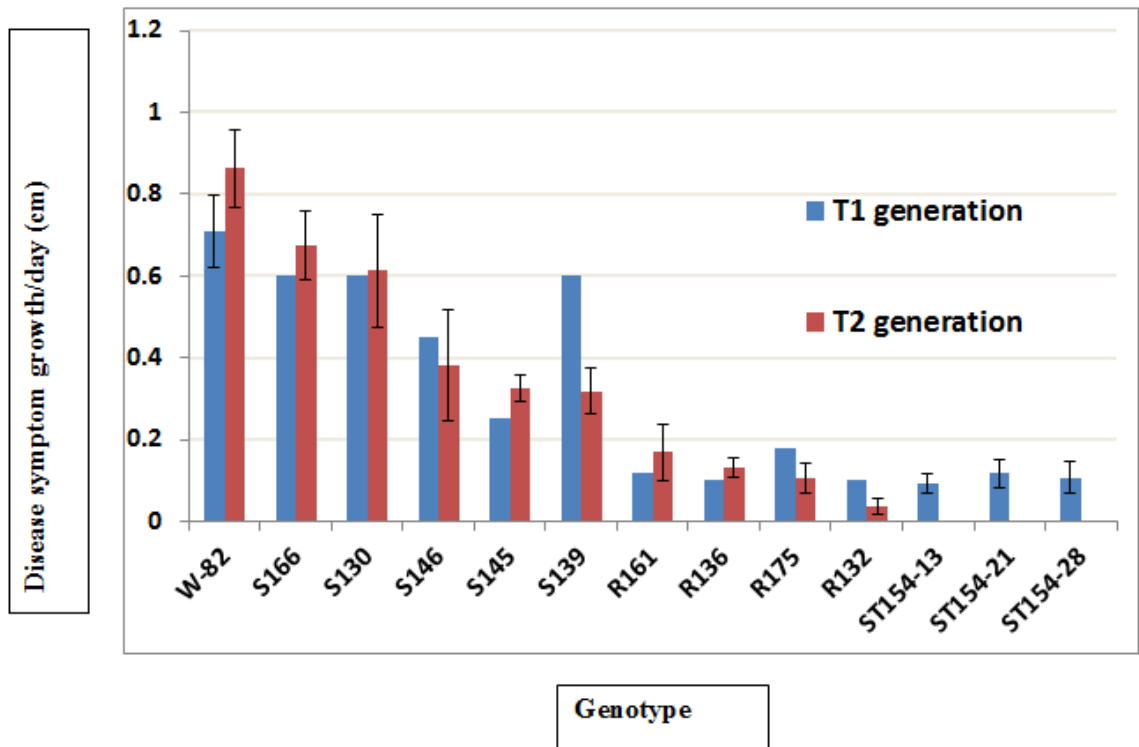


Figure Descriptions

Figure 1: Introduction of the wild-type allele of the glycine rich protein 1 (GRP1) restores resistant phenotype and nonhost resistance of the T-DNA mutant CS850460 for *GRP1*. Detached leaves of SALK mutant CS850460 plants (#5 and #7) (i) and (ii), carrying mutated, non-functional *GRP1* gene, show susceptible response while the SALK mutant lines # 5 plant #7 (iii) and line #7 plant #7 (iv), transformed with the cDNA fragment of wild type *GRP* gene, show resistant response following inoculation with *P. sojae*. The homozygous susceptible RIL *pss1-230* (v) and wild type ecotype, Columbia-0 (vi) were used as controls.

Figure 2: Arabidopsis *GRP1* restores the wild type resistant phenotype of the *pss1* mutant following *P. sojae* inoculation.

A. Leaf inoculation of *pss1* RILs 43, 230 and 463 and SALK T-DNA insertion line, CS850460 plant 5 and 7 transformed with cDNA fragment of *Arabidopsis thaliana GRP1* showed enhanced resistance following inoculation with *P. sojae* zoospores as compared to the respective untransformed control plants (10^5 zoospores/mL). The experiment was conducted two times with similar results. Error bars indicate SE. **B.** Complementation of the (i) susceptible *pss1-463* RIL plants with cDNA fragment of *Arabidopsis thaliana GRP1* gene caused recovery of the resistant phenotype in the (ii) complemented *pss1-463* RIL plants. (iii) Col-0 ecotype.

Figure 3. Characterization of SALK lines carrying T-DNA insertions in the candidate *Pss1* gene (*At3g59640*) encoding a glycine-rich protein. A) Three T-DNA lines with insertions were identified from the database search for *At3g59640*. Of them, two insertions (*grp-1* and *grp-2*) are located in the promoter and one (*grp-3*) in the first exon of *At3g59640*. Details of *grp-1*, *grp-2* and *grp-3* can be found in the Table 2. The SNP (identified from the SHORE program was found in the second exon of *At3g59640*) with an amino acid substitution from Gly to Asp is shown with an asterisk (*). PCR was performed to identify the homozygous SALK T-DNA insertion lines by using the LB; Left border primer, F; Forward primer and R; Reverse primer which can amplify both the full length fragment from WT and the T-DNA insertion. PCR was performed for B) *grp-1*, C) *grp-2*, D) and *grp-3* mutants. The homozygous lines show only the T-DNA insertion fragment but not the WT-specific band due to the large T-DNA insertion.

Figure 4. Expression analysis of the *At3g59640* gene in the homozygous SALK T-DNA insertion lines. RT-PCR was performed by using the cDNA prepared from the individual samples of *grp-1*, *grp-2* and the bulked homozygous lines of *grp-3* (carrying T-DNA insertion, details can be found in Table 2). The absence of the GRP is seen in case of the homozygous SALK T-DNA insertion lines. Actin expression was used as an internal control.

Figure 5. Enhanced broad-spectrum resistance of transgenic soybean plants carrying *Arabidopsis PSS1* to the soybean root rot pathogen, *Phytophthora sojae*. A) Leaves of the non-transgenic soybean cultivar, Williams 82, were susceptible to a *P.*

sojae race 25 (R25) (right side of the leaf); whereas, transgenic Williams 82 (ST154-13) carrying *PSSI* were resistant to that race. Note that the spread of the pathogen is localized to the inoculation sites among right side of four unifoliates. B) Reduced radial growth was recorded for three *P. sojae* isolates including R25 among two independent transgenic soybean lines (ST154-13 and ST154-21) carrying *PSSI*. The experiment was conducted twice with similar results. Error bars indicate SEs.

Figure 6. Progeny of transgenic soybean plants carrying *Arabidopsis* GRP1 gene also show enhanced resistance to soybean oomycete pathogen, *Phytophthora sojae*.

Unifoliolate leaves of 14 day old soybean plants of lines whose parents showed a susceptible (S166, S130, S146, S145 and S139) or resistant (R161, R136, R175 and R132) phenotype to *P. sojae* were inoculated with NW5A isolate of *P. sojae* spores (10^5 spores/mL) and disease spread per day was recorded. Leaves of parent transgenic lines, ST154-13, ST154-21 and ST154-28 and the non-transgenic wild type cultivar Williams-82 were included as control in the experiment. The progenies of resistant transgenic soybean lines (R161, R136, R175 and R132) showed significantly lower rates of disease symptom spread as compared to the progenies of susceptible (S166, S130, S146, S145 and S139) soybean lines. At least eight plants were inoculated for each line and the disease symptom spread was calculated by subtracting the disease lesion length of 3rd day from the 4th day post inoculation. Error bars indicate SE.

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CHAPTER 5. GENERAL CONCLUSIONS

In this study I have described the identification, cloning and characterization of a novel *Arabidopsis* nonhost resistance gene, *PSSI* which encodes a glycine rich protein (GRP1). This gene is one of several important *Arabidopsis* nonhost resistance genes involved in providing resistance against the soybean oomycete pathogen, *Phytophthora sojae*. Niederzenz (Nd) was identified as an *Arabidopsis* ecotype found to be consistently immune to *P. sojae* infection. However, due to lack of sufficient polymorphic markers between the reference genome Col-0 and Nd, a novel SBP marker technology to rapidly identify and develop polymorphic markers between these two ecotypes was developed as follows (Chapter 2). Following Illumina/Solexa sequencing of Nd genome with a ~3X coverage, the 75 bp reads of the whole genome were obtained. These reads were compared against the Col-0 reference genome using the SHORE mapping software and polymorphisms identified. Polymorphisms arising from sequencing of either at least two BAC clones or DNA molecules were considered as genuine and included in further study. About 200 bp long PCR fragments containing these polymorphisms were amplified and restriction digestion of these fragments was conducted. The digested products were run on a gel to identify the sequence based polymorphic (SBP) markers for the marker-poor regions of the genome.

In Chapter 3, the identification of a mutant *pssI* which shows lack of *Arabidopsis* nonhost resistance against two destructive soybean pathogens, *P. sojae* and *Fusarium virguliforme* is described. The *PSSI* gene was mapped to a 2.5 Mb region on the south arm of chromosome using bulked segregant analysis (BSA). This region was identified

using a SBP marker, SBP3_20.71 developed from the novel technique which has previously been described in Chapter 2. The response of *pss1* to inoculation with *P. sojae* was characterized microscopically. This investigation established that *P. sojae* became adapted to *pss1* since it was able to establish reproductive structures *in planta* in *pss1*. Following the mapping of *PSSI* gene, the *pss1* mutant was characterized for its response against a necrotrophic fungal pathogen, *F. virguliforme* and a bacterial pathogen, *Pseudomonas syringae* pv. *glycinea* (*Psg*) of soybean. The *pss1* mutant showed a loss of Arabidopsis nonhost resistance against *F. virguliforme* but not against *Psg* which cause diseases in soybean.

In Chapter 4, the map-based cloning of the Arabidopsis *PSSI* gene is presented. Following mapping of *PSSI* to a 2.5 Mb region on the south arm of chromosome 3, seven *pss1pss1* homozygous susceptible F_{2:3} were sequenced and compared to the wild type reference genome Col-0. Six candidate genes carrying non-synonymous mutations in their exons were identified from the *PSSI* region of the *pss1* mutant and SALK lines carrying T-DNA insertions in each of these genes were obtained. Following inoculation with *P. sojae*, Arabidopsis SALK T-DNA insertion lines for a *glycine rich protein 1* (*GRPI*) gene were found to show a susceptible phenotype similar to *pss1*. The transformation of both *pss1pss1* homozygous susceptible recombinant inbred lines (RILs) and the SALK T-DNA insertion lines for *GRPI* gene with cDNA sequence of wild type *GRPI* gene complemented the susceptible phenotype. Soybean cultivar, Williams 82, transformed with cDNA sequence of the Arabidopsis *GRPI* gene showed enhanced resistance against *P. sojae* isolates. Thus, I have shown that transfer of nonhost resistance mechanism is a feasible technology for enhancing disease resistance in crop plants.

Future Directions

My study has identified a novel *Arabidopsis* nonhost resistance mechanism for providing broad spectrum resistance against a destructive pathogen in soybean. I have identified *Phytophthora sojae* susceptible (*PSSI*), which encodes an *Arabidopsis thaliana* glycine rich protein (*At3g59640*, named GRP1) as a novel nonhost resistance gene which confers enhanced resistance against *P. sojae* isolates in transgenic soybean plants. Further characterization of this gene will provide useful insights into the mechanism of plant nonhost resistance and defense mechanisms. The elucidation of the subcellular localization of the GRP1 protein, both before and following inoculation with *P. sojae*, any possible basal resistance role of GRP1 against adapted pathogens of *Arabidopsis* such as *Hyaloperonospora arabidopsidis* and the specific mode of action of this novel protein are important follow-up experiments that may be conducted to characterize this novel gene.

There are recent reports of *Arabidopsis* glycine rich protein gene GRP9, being expressed specifically in root and vascular tissues [1]. The *GRP9* gene was found to be upregulated under abiotic stress such as salt stress and application of plant hormone, abscisic acid (ABA). Similarly, Shinozuka et al. [2], showed that a perennial ryegrass gene encoding a glycine rich protein is upregulated under cold stress to provide freezing tolerance. This *GRP* gene was found to express at significantly higher levels in root, crown and leaves following cold stress thus providing freezing tolerance [2].

Arabidopsis GRP1 gene may be investigated for specific spatial and temporal expression profile by conducting RT-PCR following inoculation of *P. sojae* spore

suspension and mock (water, without any spores) to establish if this gene is regulated at the transcriptional level following infection.

The sub cellular localization of a protein can be elucidated by tagging the protein with the green fluorescent protein (GFP) tag and observing the localization of the fusion protein. To investigate the localization of the Arabidopsis GRP1 protein following *P. sojae* infection, the Arabidopsis plants transformed with the GRP1-GFP fusion protein may be evaluated for green fluorescence following *P. sojae* infection. A GRP1-FLAG tag fusion protein may also be used to transform the *pss1* plants if GFP tagging renders the GRP1 protein non-functional.

RNA editing is the process of specific conversion of some cytidine residues to uridine residues and it may be required for proper protein function in plastids. In Arabidopsis, a chloroplast localized gene encoding a pentatricopeptide repeat (PPR) protein has been shown to be necessary for RNA editing in chloroplast. Glycine rich proteins (GRP) are also well known for their role in providing resistance against fungal or viral pathogens. Most of these proteins carry a signal peptide and other specific structures such as a cold shock response domain or the RNA-binding regions. The antimicrobial role of these proteins has been suggested by up-regulation of their expression upon infection with fungal pathogens. However, not much is known about the mechanism of action of these proteins in providing resistance against filamentous fungal pathogens. RNA binding property is an important mechanism of post-transcriptional regulation in higher organisms. These modifications may be brought about in the form of alternate splicing, phosphorylation, ubiquitination, nitrosylation, methylation etc. [3]. If *GRP1* carries RNA binding property, it will explain its possible role in altering fungal gene

expression to deprive the pathogen of host nutrients by preventing transcription of some of the genes important for the pathogenicity of the fungus.

This study therefore has several practical implications and may be used as a basis to not only identify other *PSS* genes in Arabidopsis, but also pioneer the successful transfer of a novel and effective nonhost resistance mechanism to a crop species of considerable economic importance.

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